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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appellant: Lara Madison, Gjalt W. Huisman and Oliver P. Peoples

Serial No.: 09/235,875 Art Unit: 1638

Filed: January 22, 1999 Examiner: Russell Kallis

For: *TRANSGENIC SYSTEMS FOR THE MANUFACTURE OF POLY(3-HYDROXY-BUTYRATE-CO-3-HYDROXYHEXANOATE)*

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

This is an appeal from the twice rejection of claims 1, 6, 7, 10, 14 and 16-21 in the Office Action mailed July 15, 2005, in the above-identified patent application. Although this Office Action is a non-final Office Action, claims 1, 6, 7, 10, 14 and 16-21 have been rejected under 35 U.S.C. § 112, first paragraph in the Office Actions mailed May 5, 2004, October 21, 2004 and July 15, 2005. A Notice of Appeal was filed on November 15, 2005. Please note that the fee for the filing of an Appeal Brief was previously submitted on April 20, 2005. A Petition for Extension of Time up to and including February 15, 2006, is enclosed along with the appropriate fee for a large entity. It is believed that no addition fee is required with this submission. However, should a fee be required, the Commissioner is hereby authorized to charge the fee to Deposit Account No. 50-3129.

(1) REAL PARTY IN INTEREST

The real party in interest of this application is the assignee, Metabolix, Inc., which has entered into licensing agreements with other parties.

(2) RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences known to appellant, the undersigned, or appellant's assignee which directly affects, which would be directly affected by, or which would have a bearing on the Board's decision in this appeal.

(3) STATUS OF CLAIMS

Claims 1, 6, 7, 10, 14 and 16-21 are pending. Claims 2-5, 8-9, 11-13, 15 and 22-34 have been cancelled. Claims 1, 6, 7, 10, 14 and 16-21 are on appeal. The text of each claim on appeal, as pending, is set forth in an Appendix to this Appeal Brief.

(4) STATUS OF AMENDMENTS

This is an appeal from the twice rejection of claims 1, 6, 7, 10, 14 and 16-21. An amendment after the non-final Office Action mailed July 15, 2005 twice rejecting the claims was filed via facsimile transmission on November 15, 2005. The claims were last amended on November 15, 2005. The Examiner has not acted on the amendment filed November 15, 2005. In a conversation with the Examiner on January 9, 2006, the Examiner stated that he would not review the Amendment and Response filed November 15, 2005 in detail before the deadline for filing an Appeal Brief. However, the Examiner indicated that the double patenting rejection would be withdrawn in light of the terminal disclaimer filed November 15, 2005. An appendix sets forth the claims on appeal.

(5) SUMMARY OF CLAIMED SUBJECT MATTER

Independent Claim 1 defines a method for the biological production of polyhydroxyalkanoate containing 3-hydroxyhexanoate, which includes providing genetically engineered bacteria expressing a thiolase gene encoding an enzyme that converts butyryl-CoA and acetyl CoA to beta-ketohexanoyl-CoA (see at least page 11, lines 19-24; page 12, lines 22-26; page 13, lines 20-26), a reductase gene that encodes an enzyme that converts beta-ketohexanoyl-CoA to beta-hydroxyhexanoyl-CoA (see at least page 12, lines 25-26; page 21, lines 18-21), and a gene that encodes a polyhydroxyalkanoate (PHA) polymerase that polymerizes 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl-CoA (see at least page 21, lines 11-15; Examples 2, 3, and 5), where the enzymes are expressed in a sufficient amount to produce polyhydroxybutyrate-co-polyhydroxyhexanoate. The polyhydroxyalkanoate (PHA) polymerase gene can be incorporated into the bacterial chromosome as defined by dependent claim 6 (see at least page 6, lines 20-21 and 26-27). As defined by dependent claim 14, the bacteria can be *E. coli* (see at least page 5, lines 13-14). In one embodiment, as defined by dependent claim 17, the bacteria expresses a PHB biosynthetic thiolase, three enzymes from *C. asetobutylicum* that form butyryl CoA, thiolase specific for 3-ketohexanoyl CoA, reductase specific for 3-ketohexanoyl CoA, and a polyhydroxyalkanoate (PHA) polymerase that accepts both 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl CoA (see at least page 12, lines 23-28). As defined by dependent claim 7, the polyhydroxyalkanoate (PHA) polymerase gene can be obtained from a bacterial strain such as *Aeromonas caviae, Comamonas testosteroni, Thiocapsia pfenigii, Chromatium vinosum, Bacillus cereus, Nocardia carolina, Nocardia salmonicolor, Rhodococcus ruber, Rhodococcus*

rhodocrous, or *Rhodospirilum rubrum* (page 3, line 19-25; page 10, line 28 to page 11, line 2; page 12, lines 1-15). The bacteria may also be engineered or selected to express a gene encoding a ß-hydroxyacyl-ACP-coenzyme A transferase as defined by dependent claim 10 (page 5, lines 20-25; page 15, lines 18-23) and/or a D-specific enoyl-CoA hydratase as defined by dependent claim 16 (page 5, lines 20-25; page 23, lines 26-29) (See Figure 5). As defined by dependent claims 18-21, the bacteria may also express one or more fatty acid biosynthetic enzymes (claim 18), such as enzymes which convert acyl ACP to acyl CoA (claim 19), including ACP-CoA transacylase, acyl ACP thioesterase, or acyl CoA synthase (claims 20 and 21) (page 15, lines 8-20; Example 6; Figures 5 and 10).

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The issues presented on appeal are:

(1) whether claims 1, 6, 7, 10, 14 and 16-21 comply with the written description requirement under 35 U.S.C. § 112, first paragraph.

(2) whether claims 1, 7, 10, 15, 18, 19 and 20 are novel as required by 35 U.S.C. § 102(b) over Timm, et al., *Appl. And Environ. Microbiol.* 56(11):3360-3367 (1990) ("Timm") and Hoffman, et al., *FEMS Microbiol. Lett.* 184:253-259 (2000) ("Hoffman").

(3) whether claims 1 and 6 are non-obvious as required by 35 U.S.C. § 103(a) over Timm in view of U.S. Patent No. 5,470,727 to Macharenas, et al. ("Macharenas"); whether claims 1, 7 14, and 16-21 are non-obvious as required by 35 U.S.C. § 103(a) over Timm in view of Schubert, et al., *J. Bacteriol.* 170(12):5837-5847 (1988) ("Schubert") and in further view of Boynton, et al., *J. Bacteriol.* 178(11):3015-3024 (1996) ("Boynton").

(7) GROUPING OF CLAIMS

The claims do not stand or fall together, as discussed below.

(8) ARGUMENT

(i) The Claimed Invention

Polyhydroxyalkanoates (PHAs) are natural, thermoplastic polyesters and can be processed by traditional polymer techniques for use in an enormous variety of applications, including consumer packaging, disposable diaper linings and garbage bags, food and medical products. Several factors are critical for economic biological production of PHAs, including substrate costs, fermentation time, and efficiency of downstream processing.

The production of PHAs containing 3-hydroxy-co-hydroxyhexanoate (3H-co-HH) in known biological systems is inefficient. However, the Appellants have discovered that microorganisms, such as *E. coli*, which do not normally produce PHAs and have not previously been described to produce 3-hydroxyhexanoate (3HH), can be genetically engineered to produce PHAs by the introduction of a PHA synthase gene which encodes an enzyme that accepts C6 substrates and optionally, additional enzyme encoding genes, such as genes encoding ß-ketothiolase, acetoacetyl-CoA reductase, ß-ketoacyl-CoA reductase, enoyl-CoA hydratase and/or ß-hydroxyacyl-ACP-coenzymeA transferase. The genes are preferably selected on the basis of the substrate specificity of their encoded enzymes for the production of 3HH polymers as well as PHB. The various pathways that can be utilized to produce PHAs are shown in Figures 2-5.

The Appellants have also discovered that biological systems for the production of PHAs containing 3-hydroxy-co-hydroxyhexanoate (3H-co-HH) can be improved by using transgenic

organisms with faster growth rates and/or by genetically engineering these organisms to produce the co-monomer 3-hydroxyhexanoic acid from cheaper feedstocks, such as butyrate or butanol, or directly from glucose by incorporating genes encoding enzymes which can channel cellular intermediates to butyryl-CoA, thereby improving the economics of PHA production using transgenic organisms. Enzyme activities desirable for conversion of metabolic intermediates into *R*-3-hydroxyhexanoyl CoA, include butyryl CoA dehydrogenase activity and acyl CoA:ACP transferase activities. The latter conversion is catalyzed either by a single protein or by a combination of thioesterase and acyl CoA synthase activities. The flux of normal cellular metabolites to 3-hydroxyhexanoate is redirected via one or more of three different pathways. These three pathways generate 3-hydroxyhexanoate, either (1) using a butyrate fermentation pathway, for example using enzymes from *Clostridium acetobutylicium* (Figure 3), (2) using fatty acid biosynthetic enzymes, for example from *E. coli* (Figure 5), or (3) using a fatty acid oxidation complex, for example, from *Pseudomonas putida* (from Figure 6). In a preferred embodiment, *E. coli* is engineered to synthesize PHBH from either inexpensive carbohydrate feedstocks such as glucose, sucrose, xylose and lactose or mixtures of such carbohydrates and fatty acids as the only carbon source by introducing genes encoding enzymes that convert cellular metabolites to 3-hydroxyhexanoyl CoA. It is crucial that the expression of all the genes involved in the pathway be adequate for efficient PHA synthesis in recombinant *E. coli* strains.

An example of a biosynthetic pathway that results in *R*-3-hydroxyhexanoyl CoA formation involves the elongation of butyryl CoA to 3-ketohexanoyl CoA which can subsequently be reduced to the monomer precursor, as shown in Figure 4. Butyryl CoA is

formed by butyrate fermenting organisms such as *C. acetobutylicum* in a four step pathway from acetyl CoA. Elongation of butyryl CoA to 3-ketohexanoyl CoA is catalyzed by a thiolase. The complete pathway thus involves (1) the PHB biosynthetic thiolase, (2) the three enzymes from *C. acetobutylicum* that form butyryl CoA, (3) a second thiolase, specific for 3-ketohexanoyl CoA, (4) a reductase specific for this substrate, and (5) a PHB polymerase that accepts both 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl CoA.

In *P. putida*, monomers for PHA biosynthesis are derived from the fatty acid oxidation pathway when alkanes or oxidized alkanes are provided as carbon and energy source. The intermediate in this pathway that is channeled to PHA biosynthesis is *S*-3-hydroxyacyl CoA (preferentially C_8 and C_{10}) which undergoes epimerization by the FaoAB complex to the *R*-isomer. The combined action of epimerase and PHA polymerase provides C_6 to C_{14} monomers for PHA. Consequently, a combination of this epimerase and a 3-hydroxyhexanoyl CoA accepting PHA polymerase provides the biosynthetic capability to synthesize PHBH from fatty acids in transgenic organisms, as shown by Figure 5. Mixtures of fatty acids and carbohydrates that are useful feedstocks for fermentative production as the 3HB monomer can be derived from acetyl CoA, where the 3HH component is from fatty acids.

P. putida and *P. aeruginosa* synthesize PHAs composed of medium-chain length 3-hydroxy fatty acids when grown on sugars. The predominant monomer in these PHAs is 3-hydoxydecanoate. A similar pathway can be engineered for the synthesis of PHBH in recombinant microorganisms such as *E. coli, R. eutropha* and *P. putida*, as shown by Figure 6. Besides a polymerase that accepts the 3-hydoxybutyryl CoA and 3-hydroxyhexanoyl CoA

precursors, an enzymatic activity that converts 3-hydroxyacyl ACP into 3-hydroxyacyl CoA or 3-ketoacyl ACP into 3-ketoacyl CoA is required. Deregulation of fatty acid biosynthesis and increased activity of this pathway subsequently provides the substrate for PHBH formation. The critical enzymatic activity in this pathway is the conversion of the 3-hydroxyacyl ACP to the CoA derivative. Thioesterases and acyl CoA synthases can accomplish this step. Alternatively, acyl ACP:CoA transferase can be used to facilitate this step in the PHA pathway.

(ii) Rejection under 35 U.S.C. § 112, first paragraph

Claims 1, 6, 7, 10, and 14-21 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor had possession of the claimed invention.

The Legal Standard

Both the written description and enablement requirements are defined by 35 U.S.C. § 112, first paragraph, which states that the patent specification must contain "a written description of the invention, and of the manner and process of making and using it...[such] as to enable any person of ordinary skill in the art to which it pertains ... to make and use the same ... " The purpose of the written description requirement is to prevent a patentee from later asserting that he invented something which he did not. Thus the patentee must "recount his invention in such detail that his future claims can be determined to be encompassed within his original creation." *Vas- Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1561, 19 U.S.P.Q.2d 1111, 1115 (Fed. Cit 1991).

The Federal Circuit has held that the written description requirement can be met by a functional description of claimed materials, if coupled with a known or disclosed correlation between function and structure. *Enzo Biochem, Inc., v. Gen-Probe, Inc.*, 296 F.3d 1316, 63 U.S.P.Q.2d 1609 (Fed. Cir.2002) ("*Enzo II*"). The court held that a patentee complied with the written description requirement by depositing biological material in a public depository. In *Enzo II*, the Federal Circuit rejected its narrow interpretation of *Eli Lilly* that the disclosure of the sequence was always necessary, and instead adopted a broader interpretation of the types of disclosures that comply with the written description requirement. The court adopted provisions from the Guidelines issued by the U.S. Patent and Trademark Office that state that the written description requirement can be met by a functional description of claimed materials, if coupled with a known or disclosed correlation between function and structure. The court found that the written description requirement was met when, in the knowledge of the art, the disclosed function is sufficiently correlated to a particular, known structure. This standard was subsequently affirmed and clarified in the decision of *Amgen Inc. v. Hoechst Marion Roussel, Inc. and Transkaryotic Therapies, Inc.* 314 F.3d 1313, 65 USPQ 2d (Fed. Cir. 2003).

There is a strong presumption that an adequate written description of the claimed invention is present when the application is filed. *In re Wertheim*, 541 F.2d 257, 263, 191 USPQ 90, 97 (CCPA 1976). An appellant shows possession of the claimed invention by **describing** the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). Possession may be

shown in many ways. For example, possession may be shown by describing an actual reduction to practice of the claimed invention. A specification may describe an actual reduction to practice by showing that the inventor constructed an embodiment or performed a process that met all the limitations of the claim and determined that the invention would work for its intended purpose. *Cooper v. Goldfarb*, 154 F.3d 1321, 1327, 47 USPQ2d 1896, 1901 (Fed. Cir. 1998). This the Appellants have clearly done.

Claims 1, 6, 7, 10, and 14 and 16-21 Satisfy the Written Description Requirement

The Examiner alleges that there is no support in claim 1 for "a *phbC* polymerase gene that encodes an enzyme that polymerizes 3-hydroxybutryl CoA and 3-hydroxyhexanoyl-CoA". Claim 1, as amended November 15, 2005 and on appeal, recites that the methods include providing genetically engineered bacteria expressing a gene that encodes a polyhydroxyalkanoate (PHA) polymerase that polymerizes 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl-CoA. The specification at least at page 4, lines, 3-8 and page 21, lines 11-15, discloses that 3-hydroxyhexanoyl CoA accepting PHA polymerase genes can be obtained from *A. caviae, C. testosteroni, T. pfenigii,* and possibly *P. denitrificans* and *S. natans*.

Further support for the polyhydroxyalkanoate polymerase gene from *A. caviae* can be found in Example 2, on page 21, lines 24-26, which cites a publication (Fukui & Doi. *J. Bacteriol.* 179:4821-4830 (1997)) (submitted with IDS filed November 4, 1999; a copy of which is enclosed in the Appendix) that describes the **sequence** for this gene. Support can also be found in Example 3, which describes the production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBH) by construction of transgenic *E. coli* strains that express

chromosomally encoded polymerase from *N. salmonicolor*. In addition, Examples 2 and 5 disclose that PHBH was produced from glucose and butyrate in *E. coli* expressing a plasmid (pMBXc12J12) containing the polymerase from *A. caviae*, which confirms that this enzyme accepts **both** 3-hydroxybutyrl CoA and 3-hydroxyhexanoyl-CoA. These examples demonstrate that enzymes that accept both 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl-CoA as substrates are not mere conjecture, but known and available to those in the art and fully identified by appellants as useful in the claimed method. Finally, Figure 9 is a schematic of selection for a PHBH recombinant pathway in *E. Coli* using the PHA polymerase gene from *P. putida*. The schematic clearly shows that the polymerase acts on 3-hydroxyhexanoyl-CoA. Accordingly, there is ample support in the specification for the claimed method and it is clear that the Appellants were in possession of the broad substrate polymerase at the time of filing.

(iii) Rejections Under 35 U.S.C. § 102

Claims 1, 7, 10, 15, 18, 19 and 20 were rejected under 35 U.S.C. § 102(b) as being anticipated by Timm in light of Hoffman.

The Legal Standard

For a rejection of claims to be properly founded under 35 U.S.C. § 102, it must be established that a prior art reference discloses each and every element of the claims. *Hybritech Inc v Monoclonal Antibodies Inc*, 231 USPQ 81 (Fed. Cir. 1986), *cert. denied*, 480 US 947 (1987); *Scripps Clinic & Research Found v Genentech Inc*, 18 USPQ2d 1001 (Fed. Cir. 1991). The Federal Circuit held in *Scripps*, 18 USPQ2d at 1010:

Invalidity for anticipation requires that all of the elements and limitations of the claim are found within a single prior art reference. . . *There must be no difference* between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention. (Emphasis added)

A reference that fails to disclose even one limitation will not be found to anticipate, even if the missing limitation could be discoverable through further experimentation. As the Federal Circuit held in *Scripps, Id.*:

[A] finding of anticipation requires that all aspects of the claimed invention were already described in a single reference: a finding that is not supportable if it is necessary to prove facts beyond those disclosed in the reference in order to meet the claim limitations. The role of extrinsic evidence is to educate the decision-maker to what the reference meant to persons of ordinary skill in the field of the invention, not to fill in the gaps in the reference.

Analysis

Claim 1

Claim 1, as amended November 15, 2005 and on appeal, specifies that the method for the biological production of polyhydroxyalkanoate containing 3-hydroxyhexanoate includes providing genetically engineered bacteria expressing a gene that encodes a polyhydroxyalkanoate (PHA) polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA.

Timm discloses transforming *Pseudomonas aeruginosa* with a polymerase (*phbC*) gene from *A. eutrophus* (*R. eutropha*). However, the first paragraph of the Discussion of Timm states

"...the PHA synthase of *A. eutrophus* exhibits a preference for CoA derivatives of hydroxyalkanoic acids with four and five carbon atoms and **does not accept** longer derivatives as substrates", ***thereby specifically excluding the claimed subject matter***. Appellants' specification also states that the polymerase from *R. eutropha* does not polymerize 3-hydroxyhexanoyl-CoA. See page 11, lines 5-9, which reads "The PHB polymerase from *R. eutropha* is highly specific for the 3-hydroxybutyryl CoA monomer and shows only 7.5% activity towards 3-hydroxyvaleryl CoA. **No** activity with **3-hydroxyhexanoyl CoA** or longer 3-hydroxyacyl CoA's was detected in *in vitro* studies (Haywood, et al., *FEMS Microbiol. Lett.*, 57:1 (1989)."

In addition, Table 1 of Timm shows that the control strain *P. aeruginosa* PAC1 synthesizes PHA containing 3-hydroxyhexanoate ***before*** the PHB-synthetic genes of *A. eutrophus* are introduced (*P. aeuruginosa* PAC1 (pVK101::PP1)- see page 3361, Results Section, lines 2-4). Therefore, the ability of this strain to produce PHA containing 3-hydroxyhexanoate is due to endogenous enzymes expressed by the strain and **not** from the expression of the *A. eutrophus* genes.

Hoffman does not make up for this deficiency. Hoffmann was cited to prove that *Pseudomonas aeruginosa* inherently comprises the *phaG* gene, i.e. a gene encoding a Beta-hydroxyacyl-ACP-coenzyme A transferase also known as ACP-CoA acyltransacylase. Hoffmann states that the phaG gene product from *P. aeruginosa* is involved in the synthesis of polyhydroxyalkanoic acid consisting of medium-chain length constituents from non-related

carbon sources. Specifically Hoffmann states that phaG exhibits 3-hydroxyacyl-ACP:CoA transacylase activity.

Since the disclosure of Timm in light of Hoffman does not disclose or suggest providing genetically engineered bacteria expressing a gene that encodes a PHA polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA, the reference does not anticipate claim 1.

Claim 7

Claim 7, dependent upon claim 1, is directed to providing a polyhydroxyalkanoate (PHA) polymerase gene from a bacteria selected from the group consisting of *Aeromonas caviae, Comamonas testosteroni, Thiocapsia pfenigii, Chromatium vinosum, Bacillus cereus, Nocardia carolina, Nocardia salmonicolor, Rhodococcus ruber, Rhodococcus rhodocrous*, and *Rhodospirilum rubrum.* Timm does not disclose or suggest providing a PHA polymerase gene from *Aeromonas caviae, Comamonas testosteroni, Thiocapsia pfenigii, Chromatium vinosum, Bacillus cereus, Nocardia carolina, Nocardia salmonicolor, Rhodococcus ruber, Rhodococcus rhodocrous*, or *Rhodospirilum rubrum.* Hoffman does not make up for this deficiency since Hoffman was cited to prove that *Pseudomonas aeruginosa* inherently comprises the *phaG* gene. Furthermore, the Examiner has not pointed to a single place in Timm that discloses providing a PHA polymerase gene from the bacteria listed in claim 7. Therefore, Timm in light of Hoffman does not anticipate claim 7.

Claims 10, 18, 19 and 20

Claim 10, dependent upon claim 1, defines the bacteria as further containing a gene encoding β-hydroxyacyl-ACP-coenzyme A transferase. Claims 18-20 define the bacteria as expressing one or more fatty acid biosynthetic enzymes; the fatty acid biosynthetic enzymes converting acyl ACP to acyl CoA; and the enzymes selected from the group consisting of ACP-CoA transacylase, acyl ACP thioesterase, and acyl CoA synthase, respectively.

As discussed above, Table 1 of Timm shows that the ability of control strain *P. aeruginosa* PAC1 to produce PHA containing 3-hydroxyhexanoate is due to endogenous enzymes expressed by the strain and **not** from the expression of the *A. eutrophus* genes.

Hoffman does not make up for this deficiency. Hoffmann was cited to prove that *Pseudomonas aeruginosa* inherently comprises the *phaG* gene, i.e. a gene encoding a Beta-hydroxyacyl-ACP-coenzyme A transferase also known as ACP-CoA acyltransacylase. Hoffmann instead states that the phaG gene product from *P. aeruginosa* is involved in the synthesis of polyhydroxyalkanoic acid consisting of medium-chain length constituents from non-related carbon sources. Specifically Hoffmann states that phaG exhibits 3-hydroxyacyl-ACP:CoA transacylase activity.

Since the disclosure of Timm in light of Hoffman does not disclose or suggest providing genetically engineered bacteria expressing a gene that encodes a PHA polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA, the reference does not disclose all of the claim elements and limitations and, thus, does not anticipate the claims.

(iv) Rejections Under 35 U.S.C. § 103

Claims 1 and 6 were rejected under 35 U.S.C. § 103(a) as obvious over Timm, in view of U.S. Patent No. 5,470,727 to Macharenas et al ("Macharenas"). Claims 1, 7 and 14-21 were rejected under 35 U.S.C. § 103(a) as obvious over Timm, in view of Schubert P. et al. *J. Bacteriol.* 170(12): 5837-5847 (1988) ("Schubert") and further in view of Boynton Z. et al. *J. Bacteriol.* 178(11): 3015-3024 (1996) ("Boynton").

The Legal Standard

References relied upon to support a rejection under 35 U.S.C. § 103 must provide an enabling disclosure, i.e., "they must place the claimed invention in the possession of the public." *Application of Payne*, 606 F.2d 303, 314, 203 U.S.P.Q. 245 (C.C.P.A. 1979); *see Beckman Instruments, Inc. v. LKB Produkter AB*, 892 F.2d 1547, 13 U.S.P.Q.2d 1301 (Fed. Cir. 1989). A publication that is insufficient as a matter of law to constitute an enabling reference may still be relied upon, but only for what it discloses. *See Reading & Bates Constr. Co. v. Baker Energy Resources Corp.*, 748 F.2d 645, 651-652, 223 U.S.P.Q. 1168 (Fed. Cir. 1984); *Symbol Technologies, Inc. v. Opticon, Inc.*, 935 F.2d 1569 (Fed. Cir. 1991).

"Focusing on the obviousness of substitutions and differences, instead of on the invention as a whole, is a legally improper way to simplify the often difficult determination of obviousness." *Gillette Co. v. S.C. Johnson & Sons, Inc.*, 919 F.2d 720, 724, 16 U.S.P.Q.2d 1923 (Fed. Cir. 1990); *see Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1383, 231 U.S.P.Q. 81, 93 (Fed. Cir. 1986). "One cannot use hindsight reconstruction to pick and choose

among isolated disclosures on the prior art to deprecate the claimed invention." *In re Fine*, 837 F.2d 1071, 1075 (Fed. Cir. 1988).

The prior art must provide one of ordinary skill in the art with the motivation to make the proposed modifications needed to arrive at the claimed invention. *See In re Geiger* , 815 F.2d 686, 2 U.S.P.Q.2d 1276 (Fed. Cir. 1987); *In re Lalu and Foulletier*, 747 F.2d 703, 705, 223 U.S.P.Q. 1257, 1258 (Fed. Cir. 1984). Claims for an invention are not *prima facie* obvious if the primary references do not suggest all elements of the claimed invention and the prior art does not suggest the modifications that would bring the primary references into conformity with the application claims. *In re Fritch*, 23 U.S.P.Q.2d, 1780 (Fed. Cir. 1992). *In re Laskowski*, 871 F.2d 115 (Fed. Cir. 1989). This is not possible when the claimed invention achieves more than what any or all of the prior art references allegedly suggest, expressly or by reasonable implication.

Obviousness is determined as follows. "A proper analysis under § 103 requires, *inter alia*, consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success." *Noelle v. Lederman*, 355 F.3d 1343, 69 USPQ2d 1508 (Fed. Cir. 2004) Both a suggestion to make a claimed composition or process and a reasonable expectation of success must be founded in the prior art, not in the appellant's disclosure. *Velander v. Garner*, 348 F.3d 1359, 68 USPQ2d 1769 (Fed. Cir. 2003); *see also In re Dow Chem. Co.*, 837 F.2d 469, 473 (Fed. Cir. 1988).

Analysis

Rejection of claims 1 and 6 over Timm, in view of Macharenas

Claim 1, as amended November 15, 2005 and on appeal, specifies that the method for the biological production of polyhydroxyalkanoate containing 3-hydroxyhexanoate includes providing genetically engineered bacteria expressing a gene that encodes a polyhydroxyalkanoate (PHA) polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA. Claim 6 specifies that the polyhydroxyalkanoate (PHA) polymerase gene is incorporated into the bacterial chromosome.

As discussed in detail above, Timm discloses transforming *Pseudomonas aeruginosa* with a gene encoding PHB polymerase from *A. eutrophus* (*R. eutropha*), which does not polymerize 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA. Macharenas merely describes the chromosomal expression of non-bacterial genes in bacterial cells.

Neither Timm nor Macharenas, alone or in combination, disclose or suggest providing genetically engineered bacteria expressing a gene that encodes a polyhydroxyalkanoate polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA. Therefore, claims 1 and 6 are not *prima facie* obvious over these references, since the references do not disclose or suggest all of the claim limitations.

Rejection of claims 1, 7, 14 and 16-21 over Timm, in view of Schubert and further in view of Boynton

Claims 1 and 16

Claim 1, as amended November 15, 2005 and on appeal, specifies that the method for the biological production of polyhydroxyalkanoate containing 3-hydroxyhexanoate includes providing genetically engineered bacteria expressing a gene that encodes a polyhydroxyalkanoate (PHA) polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA. Claim 16 specifies that the bacteria express a gene encoding a D-specific enoyl-CoA hydratase.

Both Timm and Schubert disclose introducing the PHB synthetic genes from *A. eutrophus* into *P. aeruginosa* and *E. coli*, respectively. The PHB polymerase from *A. eutrophus* does not polymerize 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA. In addition, Schubert does not describe a polymerase gene from *R. rubrum* that was available. Schubert cites to a paper that was published in 1960 (Merrick, J.M. and Doudoroff, M. Nature 189: 890-892 (1960) enclosed with the Amendment and Response filed November 15, 2005; a copy of which is enclosed in the Appendix), which only states that PHB synthase is associated with phospholipids on the surface of the PHB granules under certain conditions of growth. There is nothing in the paper about isolating, cloning, sequencing or genetically engineering a PHB synthase from *R. rubrum*.

Boynton discloses three enzymes from *C. acetobutylicum* that form butyryl CoA.

None of these references, either alone or in combination, disclose or suggest providing genetically engineered bacteria expressing a gene that encodes a PHA polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA as required by claim 1. Therefore, claim 1 is not *prima facie* obvious over these references.

Claim 7

The Examiner has failed to establish a *prima facie* case of obviousness over Timm in view of Schubert and further in view of Boynton. Timm, Schubert and Boynton do not disclose or suggest providing a PHA polymerase gene from *Aeromonas caviae*, *Comamonas testosteroni*, *Thiocapsia pfenigii*, *Chromatium vinosum*, *Bacillus cereus*, *Nocardia carolina*, *Nocardia salmonicolor*, *Rhodococcus ruber*, *Rhodococcus rhodocrous*, or *Rhodospirilum rubrum*. The Examiner has not pointed to a single place in Timm, Schubert or Boynton that discloses providing a PHA polymerase gene from the bacteria listed in claim 7. Furthermore, as stated in 35 U.S.C. § 103, the burden is on the Examiner to demonstrate that one of skill in the art would be motivated to combine these references to arrive at the bacteria listed in claim 7. The Examiner has not met his burden because the references do not disclose the bacteria listed in claim 7. Therefore, claim 7 is not *prima facie* obvious over these references.

Claim 14

Claim 14 defines the bacteria as *E. coli*. Both Timm and Schubert disclose introducing the PHB synthetic genes from *A. eutrophus* into *P. aeruginosa* and *E. coli*, respectively. The PHB polymerase from *A. eutrophus* does not polymerize 3-hydroxybutyryl CoA **and** 3-

hydroxyhexanoyl-CoA. In addition, as discussed above, Schubert does not describe a polymerase gene from *R. rubrum* that was available (see Merrick).

Boynton discloses three enzymes from *C. acetobutylicum* that form butyryl CoA.

None of these references, either alone or in combination, disclose or suggest providing genetically engineered *E. Coli* expressing a gene that encodes a PHA polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA as required by claim 14. Therefore, claim 14 is not *prima facie* obvious over these references.

Claim 17

Claim 17 defines the bacteria as expressing a PHB biosynthetic thiolase, three enzymes from *C. acetobutylicum* that form butyryl CoA, thiolase specific for 3-ketohexanoyl CoA, reductase specific for 3-ketohexanoyl CoA, and a polyhydroxyalkanoate (PHA) polymerase that accepts both 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl CoA.

As discussed above, none of these references, either alone or in combination, disclose or suggest providing genetically engineered bacteria expressing a gene that encodes a PHA polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA. Both Timm and Schubert disclose introducing the PHB synthetic genes from *A. eutrophus* into *P. aeruginosa* and *E. coli*, respectively. The specification at least at page 11, lines 10-28, describes the reductase and 3-ketothiolase activities for 3-ketohexanoyl CoA in *R. eutropha* (*A. eutrophus*) as poor. The specification states at page 11, lines 25-28 that the synthesis of 3-hydroxyhexanoyl-CoA monomers with the PHB enzymes from *R. eutropha* can be improved by using thiolase and/or reductase genes with advantageous substrate specificity for 3-ketohexanoyl-CoA. The

references do not disclose or suggest thiolases specific for 3-ketohexanoyl CoA and/or reductases specific for 3-ketohexanoyl CoA. Therefore, claim 17 is not *prima facie* obvious over these references.

Claims 18, 19, 20 and 21

Claims 18-21 define the bacteria as expressing one or more fatty acid biosynthetic enzymes; the fatty acid biosynthetic enzymes as converting acyl ACP to acyl CoA; where the enzymes are selected from the group consisting of ACP-CoA transacylase, acyl ACP thioesterase, and acyl CoA synthase; and that the enzymes are selected from the group consisting of acyl ACP thioesterase, and acyl CoA synthase, respectively.

Schubert discloses introducing the PHB synthetic genes from *A. eutrophus* into *E. coli*. Boynton discloses three enzymes from *C. acetobutylicum* that form butyryl CoA. Schubert and Boynton do not disclose or suggest any of the limitations recited in any of claims 18-21. Based on the Examiner's arguments at page 8, it appears that claims 18-21 are rejected as being obvious over Timm in view of Hoffman. Timm shows that the ability of *P. aeruginosa* to produce PHA containing 3-hydroxyhexanoate is due to endogenous enzymes expressed by the strain and **not** from the expression of the *A. eutrophus* genes. Hoffmann was cited to prove that *Pseudomonas aeruginosa* inherently comprises the *phaG* gene, i.e. a gene encoding a Beta-hydroxyacyl-ACP-coenzyme A transferase also known as ACP-CoA acyltransacylase.

As discussed above, since the disclosure of Timm in light of Hoffman does not disclose or suggest providing genetically engineered bacteria expressing a gene that encodes a PHA polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA, the reference

does not disclose all of the claim elements and limitations. Furthermore, Timm in light of Hoffman does not disclose or suggest expression of an acyl ACP thioesterase and/or acyl CoA synthase. Therefore, claims 18-21 are not *prima facie* obvious over these references.

(9) SUMMARY AND CONCLUSION

1. The claims recite methods of providing genetically engineered bacteria expressing a gene that encodes a polyhydroxyalkanoate (PHA) polymerase that polymerizes 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl-CoA. The specification at least at page 4, lines, 3-8 and page 21, lines 11-15, discloses that 3-hydroxyhexanoyl CoA accepting PHA polymerase genes can be obtained from *A. caviae, C. testosteroni, T. pfenigii,* and possibly *P. denitrificans* and *S. natans*. In addition, the Examples disclose that the polymerase from *A. caviae* (Examples 2 and 5) and the polymerase from *N. salmonicolor* (Example 3) can both form PHBH copolymers. Accordingly, it is clear from the ample support in the specification that the Appellants were in possession of polyhydroxyalkanoate polymerases with the appropriate substrate specificity and provided an enabling disclosure for one of skill in the art to use the claimed methods.

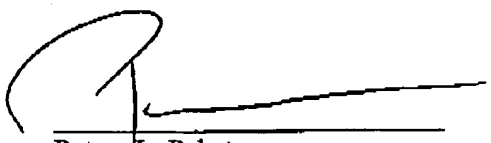
2. Timm discloses transforming *Pseudomonas aeruginosa* with a gene encoding PHB polymerase from *A. eutrophus* (*R. eutropha*), which does not polymerize 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl-CoA. Hoffman does not make up for this deficiency. Since the disclosure of Timm in light of Hoffman does not teach providing genetically engineered bacteria expressing a gene that encodes a PHA polymerase that polymerizes 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl-CoA, the reference does not disclose all of the claim elements and limitations and, thus, does not anticipate the claims.

3. Timm discloses transforming *Pseudomonas aeruginosa* with a gene encoding PHB polymerase from *A. eutrophus* (*R. eutropha*), which does not polymerize 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA. Macharenas merely teaches the chromosomal expression of non-bacterial genes in bacterial cells. Neither Timm nor Macharenas, alone or in combination, disclose or suggest providing genetically engineered bacteria expressing a gene that encodes a polyhydroxyalkanoate polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA. Therefore, the claimed methods are not *prima facie* obvious over these references.

4. Both Timm and Schubert disclose introducing the PHB synthetic genes from *A. eutrophus* into *P. aeruginosa* and *E. coli*, respectively. Boynton discloses three enzymes from *C. acetobutylicum* that form butyryl CoA. None of these references, either alone or in combination, disclose or suggest providing genetically engineered bacteria expressing a gene that encodes a PHA polymerase that polymerizes 3-hydroxybutyryl CoA **and** 3-hydroxyhexanoyl-CoA. Therefore, the claims are not *prima facie* obvious over these references, since the references do not teach or suggest all of the claim limitations.

For the foregoing reasons, Appellants submit that claims 1, 6, 7, 10, 14 and 16-21 are patentable.

Respectfully submitted,



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Claims Appendix: Claims On Appeal

1. A method for the biological production of polyhydroxyalkanoate containing 3-hydroxyhexanoate comprising providing genetically engineered bacteria expressing a thiolase gene encoding an enzyme that converts butyryl-CoA and acetyl CoA to beta-ketohexanoyl-CoA, a reductase gene that encodes an enzyme that converts beta-ketohexanoyl-CoA to beta-hydroxyhexanoyl-CoA, and a gene that encodes a polyhydroxyalkanoate (PHA) polymerase that polymerizes 3-hydroxybutyryl CoA and-3-hydroxyhexanoyl-CoA, wherein the enzymes are expressed in a sufficient amount to produce polyhydroxybutyrate-co-3-hydroxyhexanoate.

6. The method of claim 1 wherein the polyhydroxyalkanoate (PHA) polymerase gene is incorporated into the bacterial chromosome.

7. The method of claim 1 for producing a copolymer of 3-hydroxyhexanoate comprising providing a polyhydroxyalkanoate (PHA) polymerase gene from a bacteria selected from the group consisting of *Aeromonas caviae*, *Comamonas testosteroni*, *Thiocapsia pfenigii*, *Chromatium vinosum*, *Bacillus cereus*, *Nocardia carolina*, *Nocardia salmonicolor*, *Rhodococcus ruber*, *Rhodococcus rhodocrous*, and *Rhodospirilum rubrum*.

10. The method of claim 1 wherein the bacteria further comprises a gene encoding ß-hydroxyacyl-ACP-coenzyme A transferase.

14. The method of claim 1 wherein the bacteria is *E. coli*.

16. The method of claim 1 wherein the bacteria expresses a gene encoding a D-specific enoyl-CoA hydratase.

17. The method of claim 1 wherein the bacteria expresses a PHB biosynthetic thiolase, three enzymes from *C. acetobutylicum* that form butyryl CoA, thiolase specific for 3-ketohexanoyl CoA, reductase specific for 3-ketohexanoyl CoA, and a polyhydroxyalkanoate (PHA) polymerase that accepts both 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl CoA.

18. The method of claim 1 wherein the bacteria expresses one or more fatty acid biosynthetic enzymes.

19. The method of claim 18 wherein the fatty acid biosynthetic enzymes convert acyl ACP to acyl CoA.

20. The method of claim 19 where the enzymes are selected from the group consisting of ACP-CoA transacylase, acyl ACP thioesterase, and acyl CoA synthase.

21. The method of claim 20 wherein the enzymes are acyl ACP thioesterase and acyl CoA synthase.

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Cloning and Analysis of the Poly(3-Hydroxybutyrate-*co*-3-Hydroxyhexanoate) Biosynthesis Genes of *Aeromonas caviae*

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A 5.0-kbp *Eco*RV-*Eco*RI restriction fragment was cloned and analyzed from genomic DNA of *Aeromonas caviae*, a bacterium producing a copolyester of (*R*)-3-hydroxybutyrate (3HB) and (*R*)-3-hydroxyhexanoate (3HHx) [P(3HB-*co*-3HHx)] from alkanoic acids or oils. The nucleotide sequence of this region showed a 1,782-bp poly (3-hydroxyalkanoate) (PHA) synthase gene ($phaC_{Ac}$ [i.e., the *phaC* gene from *A. caviae*]) together with four open reading frames (ORF1, -3, -4, and -5) and one putative promoter region. The cloned fragments could not only complement PHA-negative mutants of *Alcaligenes eutrophus* and *Pseudomonas putida*, but also confer the ability to synthesize P(3HB-*co*-3HHx) from octanoate or hexanoate on the mutants' hosts. Furthermore, coexpression of ORF1 and ORF3 genes with $phaC_{Ac}$ in the *A. eutrophus* mutant resulted in a decrease in the polyester content of the cells. *Escherichia coli* expressing ORF3 showed (*R*)-enoyl-coenzyme A (CoA) hydratase activity, suggesting that (*R*)-3-hydroxyacyl-CoA monomer units are supplied via the (*R*)-specific hydration of enoyl-CoA in *A. caviae*. The transconjugant of the *A. eutrophus* mutant expressing only $phaC_{Ac}$ effectively accumulated P(3HB-*co*-3HHx) up to 96 wt% of the cellular dry weight from octanoate in one-step cultivation.

The utilization of biological systems for production of biodegradable materials is becoming important as a solution of the problems concerning plastic waste and the global environment. Poly(3-hydroxyalkanoates) (PHA) are produced by a wide variety of bacteria as intracellular carbon- and energy-storage materials from renewable carbon resources, such as sugars or plant oils (2, 6, 18, 25). Since these bacterial PHA are biodegradable thermoplastics, they have attracted industrial attention as possible candidates for large-scale biotechnological products. At present, more than 90 different monomeric units have been found as constituents of PHA (37).

Bacterial PHA can be divided into two groups, depending on the number of carbon atoms in the monomeric units (35). One group of bacteria, including *Alcaligenes eutrophus*, produces short-chain-length PHA with C_3-to-C_5 monomer units, while the other group, including *Pseudomonas oleovorans*, synthesizes medium-chain-length PHA with C_6-to-C_{14} monomer units. Only a few reports are available for bacteria which can synthesize PHA consisting of both short- and medium-chain-length monomer units. For example, *Rhodospirillum rubrum* (3), *Rhodocyclus gelatinosus* (19), and *Rhodococcus ruber* (12) produce terpolymers consisting of C_4, C_5, and C_6 3-hydroxyalkanoate (3HA) units from hexanoate, and some pseudomonad strains accumulate PHA consisting of C_4-to-C_{12} 3HA units (16, 38). Our laboratory has found that a random copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx), P(3HB-*co*-3HHx), is produced by *Aeromonas caviae* FA440 isolated from soil (7, 32). This bacterium synthesizes the copolyester from alkanoic acids of even carbon numbers or from plant oils up to approximately 30 wt% of the cellular dry weight, with a 3HHx fraction ranging from 10 to 25 mol%. We have also demonstrated that P(3HB-*co*-3HHx) is a flexible material and that films of the copolymer show a high degree of elongation to break (<850%) (7).

PHA biosynthesis genes, including structural genes of PHA synthases, have been isolated and analyzed at a molecular level from various sources (18, 36). The genes of *A. eutrophus* are organized in a single operon as *phbC-A-B*, which are genes of PHA synthase, β-ketothiolase, and NADPH-acetoacetyl-coenzyme A (CoA) reductase, respectively (26, 31, 34). Whereas in *P. oleovorans*, two structural genes of PHA synthases (*phaC1* and *phaC2*) flanking a PHA depolymerase gene have been identified (14), PHA synthases of *Chromatium vinosum* (21) and *Thiocapsa pfennigii* (35) consist of two different types of subunits encoded by *phbC* and *phbE* in a single operon. Although cells of *T. pfennigii* accumulated only P(3HB) homopolymer from various carbon sources, a recombinant *Pseudomonas putida* strain harboring the PHA biosynthesis genes of *T. pfennigii* produced a new type of PHA consisting of 3HB, 3HHx, and 3-hydroxyoctanoate units from octanoate (20) or of PHA containing 4-hydroxy- and 5-hydroxyalkanoate units from the related carbon sources (40, 41). In this study, we cloned and sequenced the P(3HB-*co*-3HHx) biosynthesis genes of *A. caviae* FA440 to study the molecular organization. In addition, heterologous expression of the cloned genes was examined in PHA-negative mutants of *A. eutrophus* and *P. putida* to characterize the genes and the PHA-producing ability of the recombinant strains was investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *A. caviae*, *A. eutrophus*, and *P. putida* strains were cultivated at 30°C in a nutrient-rich medium containing 10 g of meat extract, 10 g of polypeptone, and 2 g of yeast extract in 1 liter of distilled water, and *Escherichia coli* strains were grown at 37°C on a Luria-Bertani medium (28). When needed, kanamycin (50 mg/liter) or ampicillin (50 mg/liter) was added to maintain the plasmids.

DNA manipulation. Isolation of total genomic DNA and plasmids, digestion of DNA with restriction endonucleases, and transformation of *E. coli* were carried out by standard procedures (28). Transconjugation of *A. eutrophus* or *P. putida*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
A. caviae FA440	Wild type	FERM P-3432
A. eutrophus PHB⁻4	PHA-negative mutant of H16	7, 32
P. putida GPp104	PHA-negative mutant of KT2442	DSM 541, 30
E. coli		14
DH5α	*deoR endA1 gyrA96 hsdR17* (r_K^- m_K^+) *recA1 relA1 supE44 thi-1* Δ(*lacZYA-argFV169*) ϕ80Δ*lacZ*ΔM15 F⁻ λ⁻	Clontech
S17-1	*recA* and *tra* genes of plasmid RP4 integrated into the chromosome; auxotrophic for proline and thiamine	33
Plasmids		
pLA2917	Cosmid; Km^r Tc^r RK2 replicon Mob$^+$	1
pJRD215	Cosmid; Km^r Sm^r RSF1010 replicon Mob$^+$	4
pJRDEE50	pJRD215 derivative; $phaC_{Ac}$ ORF1 ORF3 ORF4 ORF5	This study
pJRDEE32	pJRD215 derivative; $phaC_{Ac}$ ORF1 ORF3	This study
pBluescriptII KS$^+$	Ap^r *lacPOZ* T7 and T3 promoter	Stratagene
pUC18	Ap^r *lacPOZ*	Takara
pEE32	pUC18 derivative; $phaC_{Ac}$ ORF1 ORF3	This study

with *E. coli* S17-1 harboring broad-host-range plasmids was performed as described by Friedrich et al. (8).

PCR. To amplify a partial fragment of the *A. caviae* PHA synthase gene from genomic DNA, we performed PCR with two primers [P1, 5'-CC(C/G)CC(C/G)TGGATCAA(T/C)AAGT(T/A)(T/C)TA(T/C)ATC-3'; P2, 5'-(G/C)AGCCA(G/C)GC(G/C)GTCCA(A/G)TC(G/C)GGCCACCA-3'] under 25 cycles of denaturation at 98°C for 20 s and annealing and elongation at 65°C for 2 min. The amplified DNA was purified by phenol extraction and ethanol precipitation.

Cloning of genes. Genomic DNA was partially digested with *Sau*3AI and ligated to the cosmid vector pLA2917 (1) linearized with *Bgl*II. The concatemeric ligation products were packaged by using Gigapack II (Stratagene), and the resultant phage particles were transfected to *E. coli* S17-1. The constructed cosmid library was screened by colony hybridization (28) with the PCR-amplified fragment as a probe to isolate the PHA biosynthesis genes of *A. caviae*. Preparation of the labeled probe and detection of the hybridization signals on membranes were carried out with a digoxigenin nucleic acid labeling and detection kit (Boehringer Mannheim).

DNA sequencing analysis. DNA fragments were subcloned into pBluescriptII KS$^-$ or pUC18, and nested sets of deletion clones were generated by using exonuclease III for DNA sequencing (28). DNA was sequenced in a DSQ-1000 DNA sequencer (Shimadzu Co., Kyoto, Japan) with a *Taq* cycle sequencing kit (Takara Co., Kyoto, Japan). Computer analysis of the resulting nucleotide sequence was performed with SDC-GENETYX genetic information processing software (Software Development Co., Tokyo, Japan).

Site-directed mutagenesis. To create restriction sites in the isolated genes, site-directed mutagenesis was carried out under the unique site elimination procedure developed by Deng and Nickoloff (5) with a U.S.E. mutagenesis kit (Pharmacia). Primers M1 and M2 (used for creation of *Bgl*II sites) and primers M3 and M4 (used for creation of *Bam*HI sites) were as follows: M1, 5'-GCCGATTGCCCCAGATCTACACTGTTCTGCC-3'; M2, 5'-GACGCTACGGGCTAGATCTCGCCTCGGGTGTG-3'; M3, 5'-CGCATGAGCGCAGGATCCCTGGAAGTAGGC-3'; and M4, 5'-GCCGTGACGGGGGGATCCGTGGTCAAGCTG-3'.

Production and analysis of PHA. One-step production of polyesters was carried out on a reciprocal shaker (130 strokes/min) at 30°C for 72 h in 500-ml flasks with 100 ml of a nitrogen-limited mineral salt medium, which was composed of 0.9 g of $Na_2HPO_4 \cdot 12H_2O$, 0.15 g of KH_2PO_4, 0.05 g of NH_4Cl, 0.02 g of $MgSO_4 \cdot 7H_2O$, and 0.1 ml of trace element solution (16). In the case of two-step production, cells were first cultivated in 100 ml of nutrient-rich medium for 12 h. Harvested and washed cells were then transferred into a nitrogen (NH_4Cl)-free mineral salt medium and incubated at 30°C for 48 h. Filter-sterilized carbon sources were added as indicated in the text. For maintenance of broad-host-range plasmids in *A. eutrophus* or *P. putida*, kanamycin was added to the medium at a concentration of 50 mg/liter. Cellular PHA content and composition were determined by gas chromatography after methanolysis of dried cells in the presence of 15% sulfuric acid, as described previously (16).

PHA synthase assay. Crude cell extracts of *A. eutrophus* transconjugants were prepared as described by Schubert et al. (31). The activity of PHA synthase was determined by spectroscopic assay according to the methods described by Valentin and Steinbüchel (43). (*R*)-3HB-CoA was synthesized by the mixed anhydride method described by Haywood et al. (11).

Enoyl-CoA hydratase assay. Recombinant *E. coli* cells were sonicated and centrifuged (20,000 × *g*, 20 min, 4°C), and the resulting soluble cell extracts were used as an enzyme solution. Enoyl-CoA hydratase activity was assayed by the hydration of crotonyl-CoA (Sigma) followed by measurement of the disappearance of absorbance at 263 nm derived from the decrease of the enoyl-thioester bond, as described by Moskowitz and Merrick (23). The configuration of 3HB-CoA produced by the hydratase was determined by coupling with the (*S*)-specific dehydrogenase. NAD^- and (*S*)-3HA-CoA dehydrogenase (Sigma) were added after the hydration reaction had reached equilibrium, and the reduction of NAD^- was monitored at 340 nm (23).

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession no. D88825.

RESULTS

Identification and cloning of the *A. caviae* PHA biosynthesis genes. For the identification of the PHA biosynthesis genes of *A. caviae*, a partial fragment of a PHA synthase gene was amplified from the genomic DNA and used as a specific probe. Two primers were designed from highly conserved regions among known PHA synthases (244-PPWINK(Y/F)YI-252 and 547-WWPDWTAWL-555; numbering corresponds to the *A. eutrophus* PHA synthase) (36), and PCR with the designed primers resulted in successful amplification of an approximately 900-bp fragment. Hybridization analysis gave only one positive signal for each of the *Eco*RI-, *Bam*HI-, *Hin*dIII-, and *Pst*I-digested genomic DNAs of *A. caviae* with a probe prepared from the amplified fragment. There were no detectable hybridization signals even under low-stringency conditions when the PHA synthase gene of *A. eutrophus* (26, 31, 34) was used as a probe. A cosmid library constructed in *E. coli* S17-1 was screened by colony hybridization, and one positive recombinant clone was isolated, which harbored a 20-kbp *A. caviae* genomic DNA fragment. A positive 11-kbp *Sal*I subfragment (SS110) was cloned into pBluescriptII KS$^+$, and further analysis showed that the PHA biosynthesis genes of *A. caviae* were located in a 5.0-kbp *Eco*RV-*Eco*RI subfragment, referred to as VE50.

Nucleotide sequence and structure of the PHA biosynthesis genes. A nucleotide sequence of the VE50 fragment was determined for both strands. Fig. 1b and 2 show the restriction map and the determined nucleotide sequence, respectively, of the VE50 fragment. Five open reading frames (ORFs) were identified (ORF1 to -5) in the fragment by computer analysis, as shown in Fig. 1c.

ORF2 (1,782 bp), which is the largest gene in this fragment, encoded a protein composed of 594 amino acids with a mo-

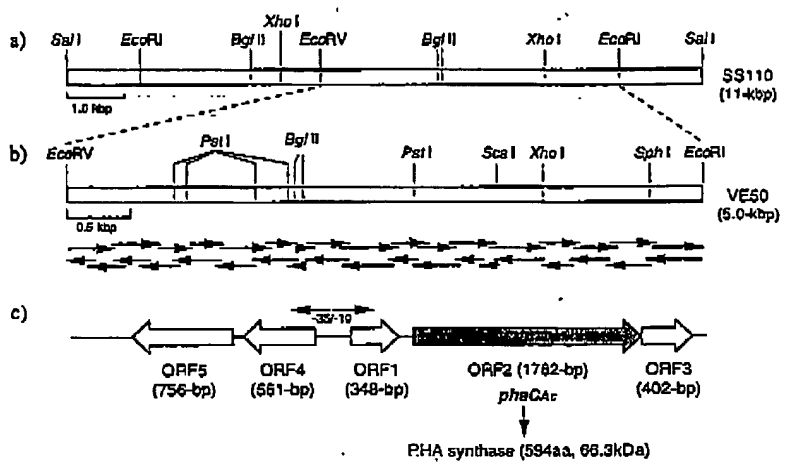

FIG. 1. Organization of *A. caviae* PHA biosynthesis genes, restriction endonuclease sites, and DNA sequencing strategy. (a) Restriction map of SS110 fragment. (b) Restriction map and sequencing strategy of VE50 subfragment. Arrows indicate sequence strategy. (c) Organization of $phaC_{Ac}$, ORF1, ORF3, ORF4, and ORF5. aa, amino acids.

lecular mass of 66,334 Da. Figure 3 shows a partial alignment and the identities of its deduced amino acid sequence with known PHA synthases from 11 microorganisms. Relatively high identities were obtained with the synthases of *Acinetobacter* sp. (45.1%) (29) and *A. eutrophus* (42.7%); therefore, ORF2 was concluded to represent a structural gene of *A. caviae* PHA synthase, and it was referred to as $phaC_{Ac}$. The calculated molecular mass of the translated PHA synthase was almost consistent with that of *A. eutrophus* (63,940 Da) and those of *P. oleovorans* (62,400 and 62,600 Da) (14). The propagation of a polyester chain has been proposed to include the formation of an acyl-*S* enzyme intermediate at two thiol groups and the transesterification to a propagating chain (17). Cys-319 in the *A. eutrophus* synthase has been demonstrated by mutagenic analysis to play important roles in the catalytic cycles (9), and the corresponding Cys residue is conserved in the PHA synthase of *A. caviae* at the same position, 319, in a lipase box-like sequence. Another active site has been proposed to be a thiol group of a 4'-phosphopantethein moiety which post-translationally modifies the synthase of *A. eutrophus* in *E. coli* (9). A candidate for the modified residue, Ser-260, is also found in the amino acid sequence deduced from $phaC_{Ac}$.

ORF1 (348 bp) and ORF3 (402 bp) were located in upstream and downstream regions, respectively, of $phaC_{Ac}$, and ORF4 (561 bp) and ORF5 (756 bp) were oriented in the opposite direction to the other three genes. Several −35 to −10 consensus sequences of a σ^{70}-dependent promoter (10) were found between ORF1 and ORF4 on both strands, suggesting that the approximately 300-bp region is a putative promoter region for these five genes. The ATG start codon of ORF3 overlapped with the TGA stop codon of $phaC_{Ac}$, and an inverted repeat, which may serve as a transcriptional termination signal, was identified in the downstream region of ORF3 (nucleotides 4899 to 4930) with the structural free energy of −166 kJ/mol. The presence of ribosome binding sequences 5 to 8 bp upstream of the ATG start codon of all genes suggests the translation of these five genes.

Complementation studies and heterologous expression. To confirm whether the cloned fragments harbor functionally active PHA biosynthesis genes, heterologous expression of the genes was investigated in the PHA-negative mutants PHB$^-$4 of *A. eutrophus* (30) and GPp104 of *P. putida* (14). The VE50 fragment (containing $phaC_{Ac}$, ORF1, ORF3, ORF4, and ORF5) and a 3.2-kbp *Bgl*II-*Eco*RI fragment (containing $phaC_{Ac}$, ORF1, and ORF3), both of which harbored the putative promoter region, were converted to *Eco*RI restriction fragments referred to as EE50 and EE32, respectively, with a p*Eco*RI linker. These two fragments were inserted into a broad-host-range vector, pJRD215 (4), at the unique *Eco*RI site, and the resultant plasmids, pJRDEE50 and pJRDEE32, were mobilized from *E. coli* S17-1 to *A. eutrophus* PHB$^-$4 or to *P. putida* GPp104. The transconjugants were cultivated in a mineral salt medium to promote the PHA biosynthesis from sugar (fructose for transconjugants of PHB$^-$4 or gluconate for those of GPp104), hexanoate, or octanoate as a carbon source.

Table 2 shows the results of PHA accumulation in the recombinant strains by one-step cultivation. The plasmids pJRDEE50 and pJRDEE32 not only could complement the deficiency of PHA synthase in the mutant strains but also could confer the ability to synthesize P(3HB-*co*-3HHx) copolymer on the hosts. *A. eutrophus* PHB$^-$4 harboring pJRDEE32 produced P(3HB) homopolymer from fructose (53 wt% of the cellular dry weight) and P(3HB-*co*-3HHx) copolymer, with 22 mol% of the 3HHx fraction from octanoate (33 wt%), while the strain harboring pJRDEE50 accumulated only a small amount of polyesters (0 to 7 wt%). *P. putida* GPp104 harboring these plasmids accumulated more than 40 wt% of P(3HB-*co*-3HHx) from hexanoate or octanoate, with a high 3HHx composition. The mole fraction of the 3HHx unit reached 40 mol% in the hexanoate-grown cells. These strains synthesized P(3HB-*co*-3HHx) not only from the carboxylic acids but also from gluconate, although the content was low (4 wt%).

Furthermore, ORF1 and/or ORF3 were deleted from the EE32 fragment, and the accumulation of PHA in transconjugants of PHB$^-$4 harboring the deleted clones was investigated. The EE32 fragment was ligated to pUC18 to form a recombinant plasmid called pEE32. Two *Bgl*II sites across the coding region of ORF1 or two *Bam*HI sites across that of ORF3 were created by site-directed mutagenesis, and elimination of the *Bgl*II or *Bam*HI fragment from the modified plasmids gave pEE32d1 and pEE32d3, respectively. pEE32d13 was also constructed by eliminating both the *Bgl*II and *Bam*HI fragments. The deleted *Eco*RI restriction fragments of these plasmids were inserted into pJRD215, resulting in the formation of

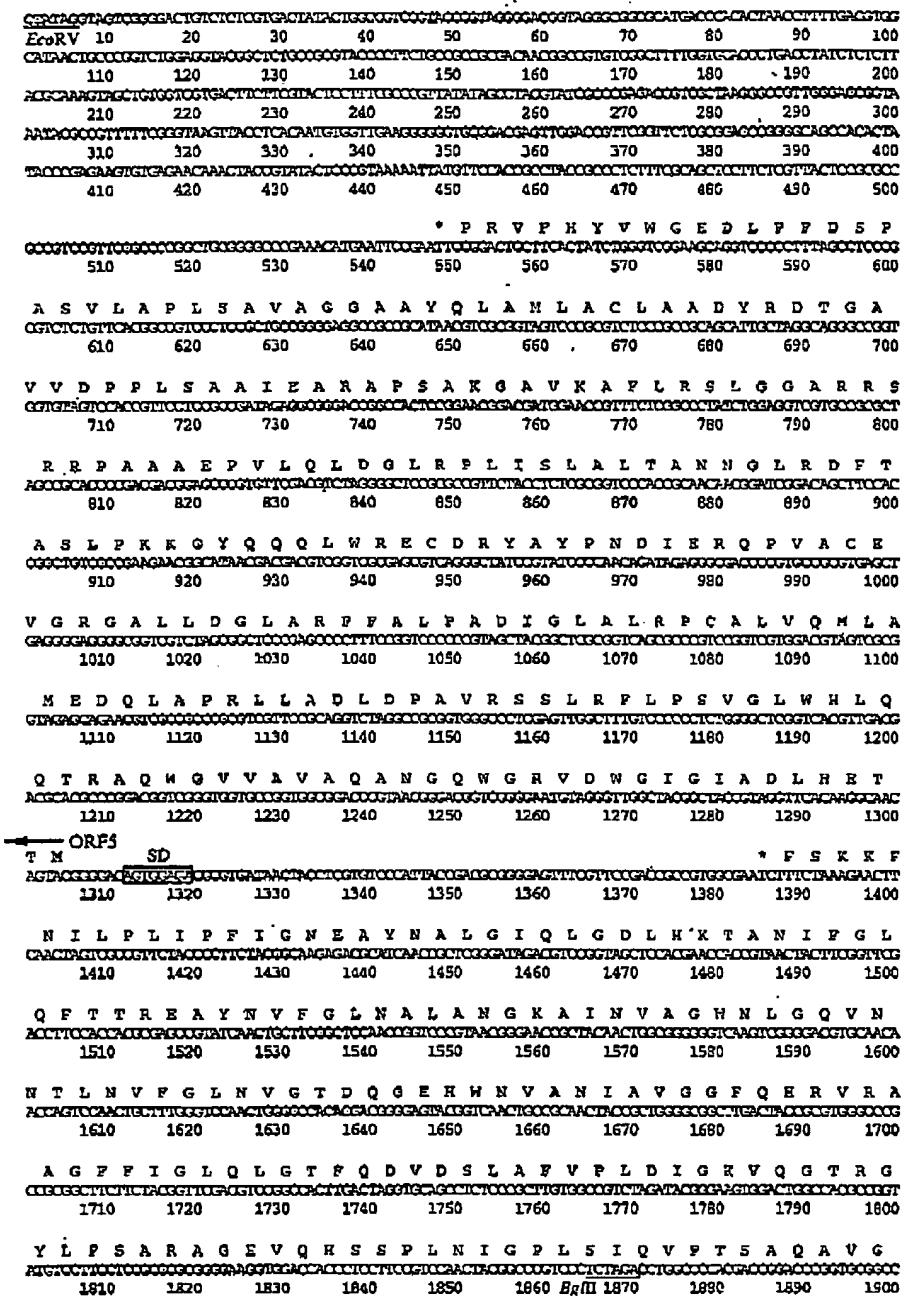

FIG. 2. Nucleotide sequence of a 5,051-bp region containing the *phaC*$_{Ae}$ gene along with the deduced amino acid sequence. The putative ribosome binding sequences are boxed and indicated by SD. The −10 and −35 regions of the σ^{70}-dependent promoter are labeled. A potential stem-loop structure is shown by the facing arrows. P1 and P2 indicate the positions of the primers used for PCR. M1, M2, M3, and M4 indicate the restriction sites created by site-directed mutagenesis. Other restriction sites are underlined and labeled. Stop codons are indicated by asterisks.

pJRDEE32d1, harboring *phaC*$_{Ae}$ and ORF3, pJRDEE32d3, harboring *phaC*$_{Ae}$ and ORF1, and pJRDEE32d13, harboring only *phaC*$_{Ae}$, *A. eutrophus* PHB^{-}4 was transformed by conjugational transfer of these recombinant plasmids, and the transconjugants were cultivated in a nitrogen-limited mineral salt medium. As shown in Table 3, all the transconjugants of PHB^{-}4 accumulated P(3HB) homopolymer from fructose, and the deletion of ORF1 and/or ORF3 slightly increased the PHA content in the cells. When the recombinant PHB^{-}4 strains were grown on octanoate or hexanoate, the content of P(3HB-*co*-3HHx) copolymer was remarkably increased by the deletion of ORFs. Especially on octanoate, the PHA content

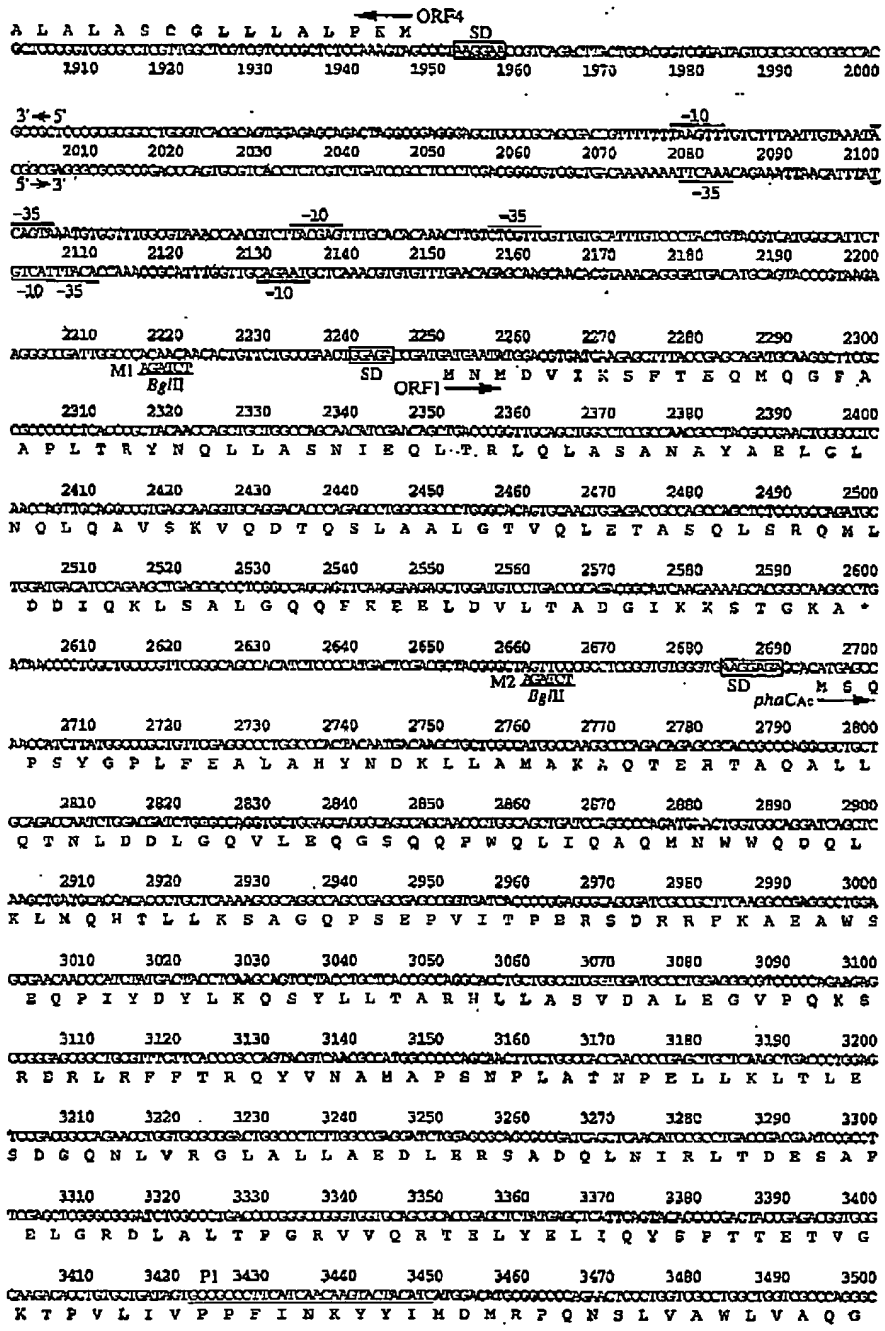

FIG. 2—*Continued.*

reached 92% of the cell weight (dry wt) after deletion of ORF1 or ORF3 from the EE32 fragment, and 96% (dry wt) after deletion of both genes. A similar tendency was also observed in the case of two-step cultivation.

Table 4 shows PHA synthase activities in the transconjugants of PHB$^-$4 at the early stationary growth phase. When octanoate was fed as a sole carbon source, the PHA synthase activity in the cells expressing $phaC_{Ac}$ with ORF1 (pJRDEE32d3) was fivefold higher than that in the cells coexpressing the three genes (pJRDEE32). Furthermore, the strain expressing only $phaC_{Ac}$ without both ORF1 and ORF3 (pJRDEE32d13) showed 38-fold higher activity than that harboring pJRDEE32. In contrast, the transconjugant harboring pJRDEE32d1 showed lower activities in spite of the high PHA content than did those harboring pJRDEE32 or pJRDEE32d3. The three strains harboring these deleted plasmids accumulated P(3HB-*co*-3HHx)

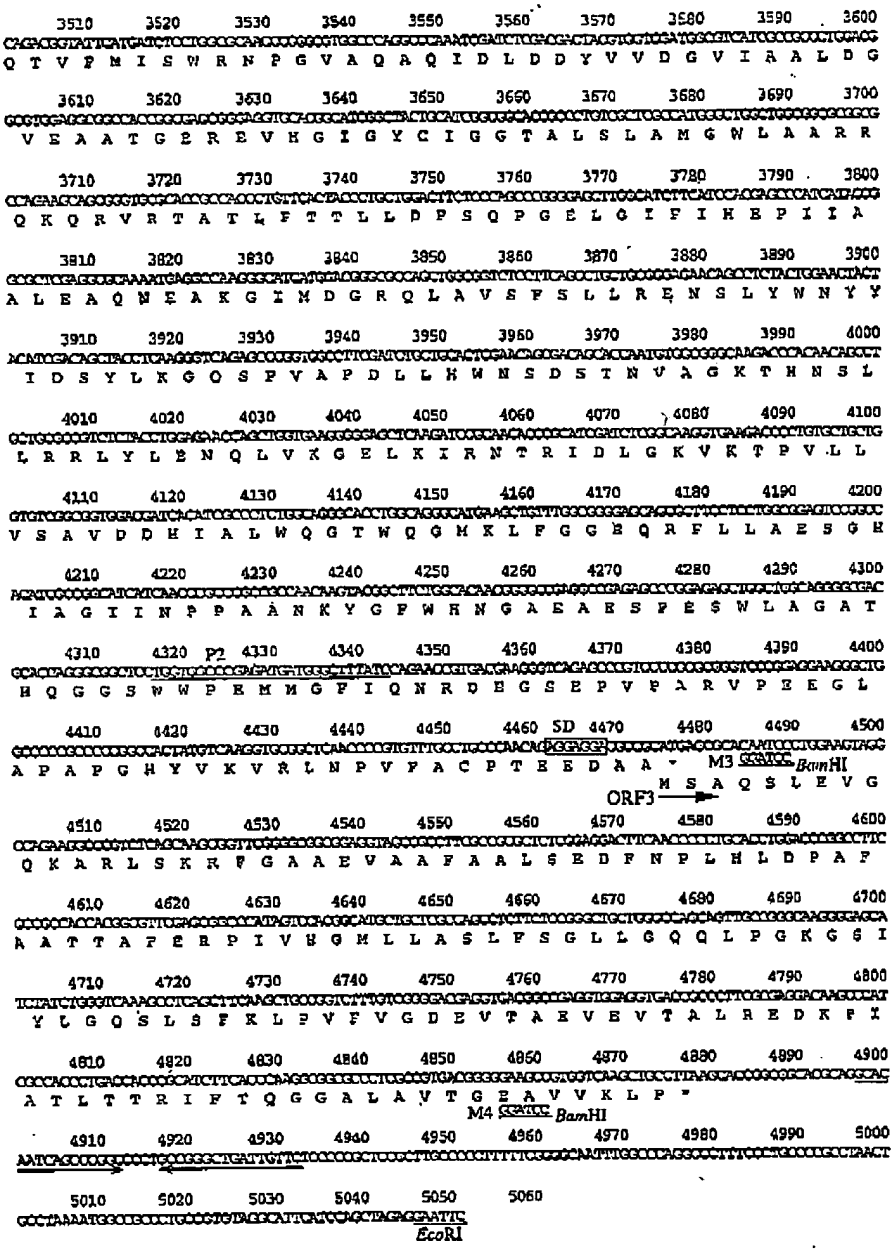

FIG. 2—*Continued.*

up to more than 92 wt% from octanoate after 72 h cultivation, but they showed quite different synthase activities. These results suggested that the PHA synthase activity does not relate directly to the PHA content accumulated in cells.

The deletion of ORFs from the EE32 fragment did not seriously affect the composition of copolyesters accumulated in octanoate-grown cells (ranging from 12 to 22 mol% of the 3HHx fraction). However, it is of interest to note that the mole fractions of the 3HHx unit in copolyesters synthesized from hexanoate were increased by the deletion of ORF3. P(3HB-*co*-3HHx), consisting of almost equimolar amounts of 3HB and 3HHx units, could be obtained by two-step cultivation of the strains harboring pJRDEE32d3 or pJRDEE32d13.

Enoyl-CoA hydratase assay. No significant identities of the amino acid sequences deduced from the nucleotide sequences of identified ORFs were detected with those of primary structures of any proteins in databases, except for the translated product of ORF3, which showed a partial identity with a putative enoyl-CoA hydratase domain of *Saccharomyces cerevisiae* β-oxidation multifunctional protein (38.4% of 73 amino acids) (13). The ORF3 product also exhibited a weak identity with *Clostridium difficile* crotonase (22.8% of 114 amino acids)

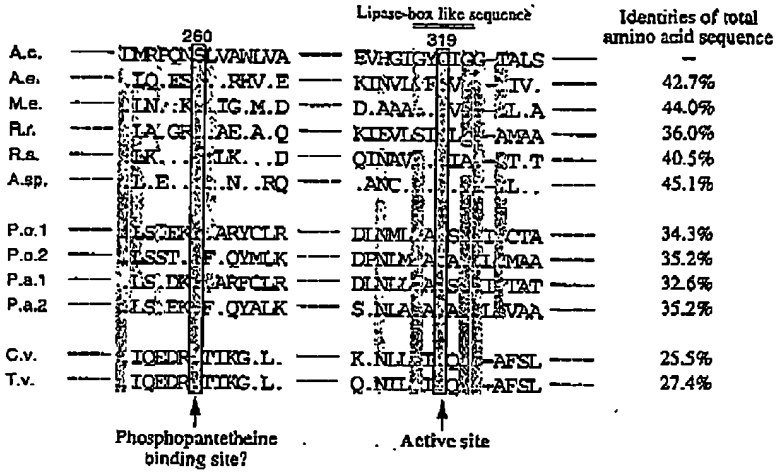

FIG. 3. Partial alignment and identities of the deduced amino acid sequence of the PHA synthase from *A. caviae* with those from *A. eutrophus* (A.e.) (26, 31, 34), *Methylobacterium extorquens* (M.e.) (42), *R. ruber* (R.r.) (27), *R. sphaeroides* (R.s.) (15), *Acinetobacter* sp. (A.sp.) (29), *P. oleovorans* (P.o.1 and P.o.2) (14), *P. aeruginosa* (P.a.1 and P.a.2) (39), *C. vinosum* (C.v.) (21), and *Thiocystis violacea* (T.v.) (22). Dots, amino acids identical to the *A. caviae* sequence; shading, amino acids which are identical in at least eight PHA synthases.

(24). Therefore, soluble extracts of recombinant *E. coli* harboring the PHA biosynthetic genes of *A. caviae* were prepared, and enoyl-CoA hydratase activity was assayed with crotonyl-CoA as a substrate. The results are given in Table 5. *E. coli* harboring pEE32 or pEE32d1 exhibited a high enoyl-CoA hydratase activity, while the activity in the cells harboring pEE32d3 or pEE32d13 was as low as that in a control strain harboring pUC18. To investigate the stereospecificity of the hydration reaction, NAD^+ and (*S*)-3HA-CoA dehydrogenase were added to the reaction mixture after the hydration of crotonyl-CoA had reached equilibrium (23), but the formation of NADH linked with the oxidation of (*S*)-3HB-CoA was not observed. These results strongly suggest that ORF3 is a structural gene of (*R*)-specific enoyl-CoA hydratase. The β-oxidation multifunctional protein of *S. cerevisiae* encoded by the *fox2* gene has been reported to catalyze the hydration of enoyl-CoA with (*R*)-specificity (13). The evolutionary relationship between *A. caviae* ORF3 and *S. cerevisiae fox2* may be an interesting subject.

DISCUSSION

There are only a few studies on bacteria capable of incorporating both short- and medium-chain-length 3HA units into polyester chains. *R. ruber* (12) and *R. rubrum* (3) are known to synthesize PHA with C_4-to-C_6 3HA units, and the PHA synthase genes of these bacteria have been cloned (15, 27). However, the substrate specificities of the translated PHA synthases were not investigated thoroughly. In this study, cloning of PHA biosynthesis genes, including a structural gene of PHA synthase from *A. caviae* ($phaC_{Ac}$), was performed together with an investigation of the PHA-producing ability of recombinant strains harboring the cloned genes.

A partial fragment of a PHA synthase gene was successfully amplified from genomic DNA of *A. caviae* by PCR with primers designed from a highly conserved region among various PHA synthases. The amplified fragment was then used as a specific probe for identification and isolation of the PHA biosynthesis genes of *A. caviae*. These PCR primers are expected to be useful for cloning of PHA synthase genes from other bacteria.

The nucleotide sequence indicated that $phaC_{Ac}$ was clustered with four ORFs (ORF1, -3, -4, and -5) and one putative promoter region in a 5.0-kbp genomic fragment. The deduced amino acid sequence of *A. caviae* PHA synthase shows 42.7% identity with the synthase of *A. eutrophus* (specific for short-chain-length 3HA), which is higher than its 32.6 to 35.2% identity with those of pseudomonads (specific for medium- and

TABLE 2. Accumulation of PHA in recombinant strains harboring PHA biosynthesis genes of *A. caviae*[a]

Strain	Plasmid	Carbon source	PHA content (wt%)	Composition (mol%)	
				3HB	3HHx
A. eutrophus PHB⁻4	pJRD215	Fructose	0		
		Hexanoate	0		
		Octanoate	0		
	pJRDEE50	Fructose	7	100	0
		Hexanoate	Trace		
		Octanoate	6	96	4
	pJRDEE32	Fructose	53	100	0
		Hexanoate	6	83	17
		Octanoate	33	78	22
P. putida GPp104	pJRD215	Gluconate	0		
		Hexanoate	0		
		Octanoate	0		
	pJRDEE50	Gluconate	4	71	29
		Hexanoate	42	61	39
		Octanoate	41	71	29
	pJRDEE32	Gluconate	4	71	29
		Hexanoate	38	60	40
		Octanoate	48	69	31

[a] Cells were cultivated in a nitrogen-limited mineral salt medium containing fructose (0.5% wt/vol), the sodium salts of gluconate (1.5% wt/vol), and octanoate or hexanoate (0.1% wt/vol × 5 for *A. eutrophus* strains or 0.5% wt/vol for *P. putida* strains) as a sole carbon source for 72 h at 30°C.

TABLE 3. Accumulation of PHA in recombinant strains of *A. eutrophus* PHB⁻4 harboring deleted clones of PHA biosynthesis genes of *A. caviae*

Plasmid (relevant markers)	Carbon source	One-step cultivation[a]			Two-step cultivation[b]		
		PHA content (wt%)	Composition (mol%)		PHA content (wt%)	Composition (mol%)	
			3HB	3HH		3HB	3HHx
pJRDEE32 (*phaC*$_{Ac}$, ORF1, ORF3)	Fructose	53	100	0			
	Hexanoate	6	83	17	4	76	24
	Octanoate	33	78	22	6	85	15
pJRDEE32d1 (*phaC*$_{Ac}$, ORF3)	Fructose	66	100	0			
	Hexanoate	78	84	16	25	77	23
	Octanoate	92	87	13	53	85	14
pJRDEE32d3 (*phaC*$_{Ac}$, ORF1)	Fructose	66	100	0			
	Hexanoate	44	75	25	19	53	47
	Octanoate	92	88	12	23	87	13
pJRDEE32d13 (*phaC*$_{Ac}$)	Fructose	73	100	0			
	Hexanoate	72	72	28	26	50	50
	Octanoate	96	85	15	50	80	20

[a] Cells were cultivated in a nitrogen-limited mineral salt medium containing fructose (0.5% wt/vol) and sodium hexanoate or sodium octanoate (0.1% wt/vol × 5) as a sole carbon source for 72 h at 30°C.
[b] Cells were grown in a nutrient-rich medium for 12 h at 30°C, transferred into a nitrogen-free mineral salt medium containing sodium hexanoate or sodium octanoate (0.25% wt/vol × 2) as a carbon source, and incubated for 48 h at 30°C.

long-chain-length 3HA). Although *R. ruber* synthesizes copolyesters of C_4 to C_6 units similar to those of *A. caviae* (12), the PHA synthase of *R. ruber* has closer identity to the synthases of *Pseudomonas aeruginosa* than to those of *A. eutrophus* and *A. caviae*. It is difficult to predict the substrate specificity of PHA synthases on the basis of their primary structures.

Heterologous expression of *phaC*$_{Ae}$ in the PHA-negative mutants PHB⁻4 of *A. eutrophus* and GPp104 of *P. putida* resulted in the accumulation of P(3HB-*co*-3HHx) copolyesters from hexanoate or octanoate and of P(3HB-*co*-3-hydroxyvalerate) from pentanoate (data not shown). A 3-hydroxyoctanoate unit was never detected, even in the copolyesters produced by transconjugants of GPp104 from octanoate. These results indicate that the *A. caviae* PHA synthase is active toward C_4-to-C_6 3HA-CoA and that the composition of the copolyester produced by *A. caviae* FA440 reflects the substrate specificity of the PHA synthase. The acceptance of 3HHx-CoA as a substrate is a significant difference between the *A. caviae* PHA synthase and other synthases, being specific for short-chain-length C_3-to-C_5 3HA-CoA only.

E. coli strains expressing the ORF3 gene of *A. caviae* showed (*R*)-specific enoyl-CoA hydratase activity, suggesting that the translated product of ORF3 functions as an enzyme in a monomer-supplying pathway for PHA biosynthesis. Figure 4 shows a proposed PHA biosynthesis pathway in *A. caviae*. Acyl-CoA derived from alkanoic acids or oils is degraded via cyclic β-oxidation, resulting in the formation of enoyl-CoA intermediates of different chain lengths. These intermediates may be converted to (*R*)-3HA-CoA by the (*R*)-specific enoyl-CoA hydratase encoded by ORF3, and the resultant (*R*)-3HA-CoA of 4 to 6 carbon atoms may be incorporated into a growing polyester chain by the function of PHA synthase. Hence, P(3HB-*co*-3HHx) is synthesized from alkanoic acids of even carbon numbers or from oils. Small amounts (5 mol% ± 2 mol%) of

TABLE 4. PHA synthase activity and PHA accumulation in recombinant strains of *A. eutrophus* at the early stationary growth phase[a]

Strain	Plasmid (relevant markers)	Carbon source	PHA synthase[b] (U/g of protein)	PHA content (wt%)	Composition (mol%)	
					3HB	3HHx
H16	pJRD215	Fructose	138	60	100	0
		Octanoate	61	67	100	0
PHB⁻4	pJRD215	Fructose	5	0		
		Octanoate	3	0		
PHB⁻4	pJRDEE32 (*phaC*$_{Ae}$, ORF1, ORF3)	Fructose	145	25	100	0
		Octanoate	20	12	61	39
PHB⁻4	pJRDEE32d1 (*phaC*$_{Ae}$, ORF3)	Fructose	33	49	100	0
		Octanoate	13	51	88	12
PHB⁻4	pJRDEE32d3 (*phaC*$_{Ae}$, ORF1)	Fructose	110	28	100	0
		Octanoate	114	37	85	15
PHB⁻4	pJRDEE32d13 (*phaC*$_{Ae}$)	Fructose	425	69	100	0
		Octanoate	770	69	85	25

[a] Cells were cultivated in a nitrogen-limited mineral salt medium containing fructose (0.5% wt/vol) or sodium octanoate (0.1% wt/vol × 3) as a sole carbon source for 30 h at 30°C.
[b] PHA synthesis activity for (*R*)-3HB-CoA.

TABLE 5. Enoyl-CoA hydratase activity in recombinant strains of *E. coli* DH5α[a]

Plasmid	Relevant markers	Enoyl-CoA hydratase[b] (U/g of protein)
pUC18		39
pEE32	$phaC_{Ac}$, ORF1, ORF3	617
pEE32d1	$phaC_{Ac}$, ORF3	323
pEE32d3	$phaC_{Ac}$, ORF1	31
pEE32d13	$phaC_{Ac}$	46

[a] Cells were cultivated in a Luria-Bertani medium for 24 h at 37°C.
[b] Hydration activity for crotonyl-CoA.

a 3HB unit were reported to be incorporated into a copolyester with a 3-hydroxyvalerate unit (95 mol% ± 2 mol%) by *A. caviae* from alkanoic acids of odd carbon numbers (7). In *A. caviae*, there are low activities of β-ketothiolase (0.09 U/mg of protein) and NADH-acetoacetyl-CoA dehydrogenase (0.06 U/mg of protein), but no activity of NADPH-acetoacetyl-CoA reductase was detected. The small amount of a 3HB unit in the copolyesters may be supplied from two acetyl-CoA molecules via four-step reactions catalyzed by β-kerothiolase, NADH-acetoacetyl-CoA dehydrogenase, crotonase [(*S*)-specific enoyl-CoA hydratase], and (*R*)-specific enoyl-CoA hydratase (Fig. 4), which is similar to the pathway in *R. rubrum* (23). The deletion of ORF3 from the EE32 fragment resulted in an enrichment of the 3HHx fraction in P(3HB-*co*-3HHx) produced by the recombinant strains of *A. eutrophus* PHB⁻4 (Table 3). This result may suggest the major contribution of the ORF3 product in supplying a 3HB unit; that is, the (*R*)-specific enoyl-CoA hydratase encoded by ORF3 may be more specific for the hydration of crotonyl-CoA than for that of medium-chain-length enoyl-CoA.

The transconjugant of *A. eutrophus* PHB⁻4 harboring pJRDEE50 accumulated a smaller amount of polyesters than that harboring pJRDEE32, while there was little difference in the PHA content and composition between transconjugants of *P. putida* GPp104 harboring pJRDEE50 and those harboring pJRDEE32. The functions of ORF4 and ORF5 in PHA biosynthesis are still unknown. The deletion of ORF1 and/or ORF3 from the EE32 fragment drastically increased the P(3HB-*co*-3HHx) content in the cells of recombinant PHB⁻4 when hexanoate or octanoate was fed as a sole carbon source. Here, it is noted that a PHA content of 96 wt% was achieved in the strain harboring pJRDEE32d13 from octanoate. The coexpression of ORF1 and ORF3 with $phaC_{Ac}$ seems to lead to a decrease in intracellular PHA content. Enzymatic analysis revealed that the deletion of the surrounding genes also affected the activity of PHA synthase. However, the PHA content accumulated in cells was not correlated to the level of PHA synthase activity (Table 4). For example, the PHA content in octanoate-grown cells harboring pJRDEE32d13 was only slightly higher than that of cells harboring pJRDEE32d1 at both the early stationary (30 h) and late stationary (72 h) phases, even though the former strain showed 60-fold higher synthase activity than the latter. In addition, the effects of the deletion of the ORF3 gene on the activity of PHA synthase and on the cellular content of PHA cannot be explained by the catalytic function of the translated product, (*R*)-specific enoyl-CoA hydratase. Further studies, including the investigation of transcriptional efficiency of each transconjugant, will be necessary to elucidate the characters and functions of these ORFs.

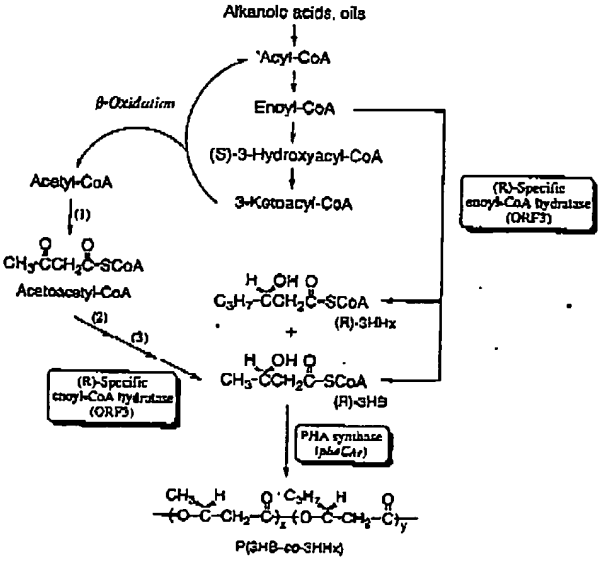

FIG. 4. Proposed pathway of P(3HB-*co*-3HHx)-biosynthesis in *A. caviae* from alkanoic acids or oils. (1) β-Ketothiolase; (2) NADH-acetoacetyl-CoA dehydrogenase; (3) crotonase [(*S*)-specific enoyl-CoA hydratase].

Liebergesell et al. (20) have reported that PHA biosynthesis genes of the anoxygenic phototrophic bacteria *C. vinosum*, *Rhodobacter sphaeroides*, and *T. pfennigii* conferred the ability to synthesize P(3HB-*co*-3HHx) from octanoate on *P. putida* GPp104. It is interesting that the PHA synthases of *C. vinosum* and *T. pfennigii* exhibited similar specificity to that of *A. caviae* in spite of their quite different structures. They have also reported that P(3HB) homopolymer was accumulated in the octanoate-grown cells of *A. eutrophus* PHB⁻4 harboring the genes of *T. pfennigii*, in contrast to the production of PHA containing nearly a half-molar ratio of the 3HHx fraction by the GPp104 strain expressing the same genes. In this study, the transconjugant of PHB⁻4 harboring pJRDEE32d13, expressing only $phaC_{Ac}$ as a foreign gene, efficiently accumulated P(3HB-*co*-3HHx) from hexanoate or octanoate, and the 3HHx fraction in the copolyester reached 50 mol% in the recombinant cells from hexanoate by two-step cultivation. Apparently, *A. eutrophus* cells have the ability to supply the (*R*)-3HHx-CoA thioester intermediate from these carboxylic acids. The P(3HB) accumulation from octanoate in recombinant PHB⁻4 strains described by Liebergesell et al. may be caused by the effects of additional genes in the cloned fragment which were introduced into the host together with the PHA synthase gene.

In conclusion, this study not only gives information on PHA biosynthesis genes of *A. caviae* but also demonstrates the usefulness of the recombination techniques for an efficient production of copolyesters consisting of short- and medium-chain-length 3HA-CoA. Further studies will be done to clarify the mechanism of synthesis and accumulation of PHA in *A. caviae* and in recombinant strains harboring the PHA biosynthesis genes of *A. caviae*.

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Table 2. Development of Active Sensitization to Tuberculin in Guinea Pigs receiving Disrupted Cells from Peripheral Blood, Spleen, and Lymph Nodes of Hypersensitive Donors

		Days after injection of cells						
		0	7	13–14	17–25	33–43	61–65	82–85
Peripheral leucocytes (~1 × 10⁸ cells)								
	No. tested	3	6	8	5	11	8	8
(a)	Strong reactions*	0	0	0	2	4	4	3
	Weak reactions	0	1	4	1	4	1	1
	Anaphylaxis†							7 (3 died)
Spleen (~3 × 10⁸ cells)								
	No. tested	3	6	8	8	12	9	9
(b)	Strong reactions	0	0	0	0	0	2	4
	Weak reactions	0	1	4	8	7	1	3
	Anaphylaxis							8 (6 died)
Lymph node cells (~5 × 10⁸ cells)								
	No. tested	—	—	6	3	6	3	3
(c)	Strong reactions	—	—	0	1	1	1	1
	Weak reactions	—	—	4	0	2	0	1
	Anaphylaxis							2 (1 died)
No cells								
	No. tested	3	9	—	6	—	6	6
(d)	Strong reactions	0	0	—	0	—	0	0
	Weak reactions	0	1	—	0	—	0	2
	Anaphylaxis							2 (none died)

* Strong reactions were >10 × 10 mm.; weak reactions 7 × 7 to 10 × 10 mm. † Tested with 2 mgm. *PPD* intravenous.

stage or other and only 2 out of the 18 animals, which were followed over the whole period, failed to show any reaction. As a control, 9 normal animals were skin tested with *PPD* 5 times over a similar period. One of these showed reactivity on the second injection 7 days after the first, and afterwards failed to react, and 2 others showed very mild reactions which appeared only at the end of the three months course. When frozen and thawed peripheral blood leucocytes and spleen cells from normal unsensitized animals were used the recipients behaved like the control group with no cells.

At the end of the experiment all four groups were challenged with 2 mgm. *PPD* intravenously. Sixteen out of 18 animals which had received disrupted cells from hypersensitive donors developed anaphylaxis and 10 died. Only 2 of the 9 controls developed very mild anaphylaxis.

From these results it appears that a transient delayed hypersensitivity is transferred by intact peripheral blood leucocytes, spleen cells and lymph node cells, and that it may be followed by a superimposed active immunization which persists far beyond the time when the donor cells would have been destroyed by a homograft reaction. Peripheral blood leucocytes were more effective in respect of active sensitization than cells from the spleen and lymph nodes. A further property of cells from sensitized donors is that they can enhance the development of antibody against tuberculin accompanying multiple injections of *PPD*. *PPD* is not a single substance, and there is no evidence whether the antibody is directed against traces of contaminating antigen which are known to be present.

These experiments indicate that the possibility that active immunization has occurred must be borne in mind in the interpretation of experiments on passive transfer of delayed hypersensitivity.

I would like to thank Dr. J. H. Humphrey for his advice throughout this investigation.

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ENZYMATIC SYNTHESIS OF POLY-β-HYDROXYBUTYRIC ACID IN BACTERIA

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THE polymeric ester, poly-β-hydroxybutyric acid, originally discovered by Lemoigne in 1927 as a major component of *Bacillus megaterium*, has since been found to be an important storage material in a variety of bacteria[1–4]. It serves as an intracellular reserve of carbon and reducing power in the photosynthetic bacterium, *Rhodospirillum rubrum*, and as the principal substrate for the endogenous respiration of certain aerobes[3–6].

The present report deals with the synthesis of the polymer from carbon-14-labelled D(−)-β-hydroxybutyryl-coenzyme *A* by particulate fractions of cell-free extracts from *B. megaterium* and *R. rubrum*.

Radioactive D(−)-β-hydroxybutyric acid was obtained by alkaline hydrolysis of purified polymer isolated from cells of *R. rubrum* that had assimilated uniformly labelled acetate in the light[3]. The acid was purified by paper chromatography, and the coenzyme *A* derivative was prepared by the mixed anhydride method of Wieland and Rueff[6]. Free β-hydroxybutyric acid was removed from the acidified solution by ether extraction. Analysis of the specific

Table 1. SYNTHESIS OF POLYMER BY *B. megaterium* EXTRACTS

Time (min.)	Percentage carbon-14 recovered in polymer fraction Exp. 1	Exp. 2	Exp. 3
0	0	0	0
5	—	27	—
10	—	35	—
20	33	45	46
40	36	44	—

The reaction mixtures contained either recombined particulate and supernatant fractions (1·08 mgm. polymer and 1·75 mgm. protein per ml.) or resuspended particulate fraction alone (1·08 mgm. polymer and 0·13 mgm. protein per ml.); and the following components (in μmoles per ml.): magnesium chloride, 12·5; glutathione, 12·5; and potassium phosphate buffer, *p*H 7·4, 125.

Exp. 1: particulate + supernatant fractions + 0·48 μequiv. D(−)-β-hydroxybutyryl-coenzyme *A* per ml. (initial); Exps. 2 and 3: particulate fraction + 0·24 and 0·48 μequiv. D(−)-β-hydroxybutyryl-coenzyme *A* per ml. (initial), respectively.

The reaction mixtures were incubated at 33° C.

activity of the product, coupled with hydroxamate and sulphydryl determinations, indicated that at least 95 per cent of the acid was present in thioester combination. Since the commercially available coenzyme *A* used for the synthesis is only 50–75 per cent pure and is contaminated with other sulphydryl compounds (for example, glutathione), the assays reflect the total concentration of β-hydroxybutyryl thioesters, rather than of the coenzyme *A* derivative alone. Poly-β-hydroxybutyric acid was assayed with a modified hydroxamate method for ester determination[7], or by the spectrophotometric method of Slepecky and Law[8].

B. megaterium, strain Km, was grown in the medium containing 2 per cent glucose described by Macrae and Wilkinson[4]. The cells were gathered at the end of exponential growth and washed with 0·033 *M* phosphate buffer, *p*H 7·0. Cell-free extracts were prepared by the osmotic lysis of concentrated protoplasts made by lysozyme treatment of the cells[4]. The lysates were subjected to sonic oscillation to liberate the polymer particles from the cell membranes. The heavy particulate fraction (consisting mainly of polymer particles) was separated from the extract by centrifugation at 3,000 *g* for 20 min. at 4° C., and the particles were washed several times with phosphate buffer, *p*H 7·0. The supernatant fraction contained both the soluble proteins of the cell and the disintegrated cell membranes.

The particulate fraction was incubated with radioactive β-hydroxybutyryl-coenzyme *A* in the presence and absence of the supernatant fraction. The reaction was stopped by addition of alcohol (60 per cent final concentration) and the radioactivity of the isolated polymer was measured. The particles were sedimented by centrifugation, treated with alkaline hypochlorite, washed successively with water and acetone and finally dissolved in chloroform[1,4]. Suitable aliquots were analysed for radioactivity. Controls were performed with reaction mixtures in which β-hydroxybutyryl-coenzyme *A* was replaced by labelled β-hydroxybutyrate in the presence or absence of coenzyme *A*, as well as by alkali-hydrolysed coenzyme *A* derivative. No incorporation of radioactivity into polymer was observed in the control reaction mixtures. In the presence of β-hydroxybutyryl-coenzyme *A*, on the other hand, the polymer became rapidly labelled with carbon-14 when the particulate fraction was used either alone or in combination with the supernatant fraction (Table 1).

The reaction came to completion in about 20 min., at which time 35–45 per cent of the total radioactivity was recovered in the polymer. The observed maximal conversion of thioester to polymer does not give any information on the equilibrium constant for the synthesis of polymer from β-hydroxybutyryl-coenzyme *A*, since the true concentration of the latter compound was not known for reasons previously discussed.

To study the stoichiometry of the reaction, a further experiment was performed in which the disappearance of thioester was measured. The analyses showed that in a reaction mixture initially containing 1·2 mgm. washed polymer particles (without supernatant fraction) and 1·2 μequiv. of thioester per ml., 0·5 μequiv. of β-hydroxybutyrate was incorporated in the polymer, while 0·6 μequiv. of thioester had disappeared. Thus, the synthesis of polymer accounted for more than 80 per cent of the thioester decomposed.

To identify the product unequivocally, the labelled polymer from the above experiment was diluted with purified carrier polymer[1,4] and reprecipitated three times from chloroform: twice with 95 per cent ethanol and once with acetone–ether (2 : 1). The specific activity remained constant throughout. The reprecipitated polymer was hydrolysed for 22 hr. at 30° in *N* potassium hydroxide, treated with cation exchange resin ('Dowex 50', H^+), and chromatographed on paper using the following solvents: (1) ether–benzene–formic acid–water (50 : 50 : 12·5 : 2); (2) *n*-butanol–glacial acetic acid–water (100 : 6 : 25); (3) *n*-butanol saturated with 1·5 *M* ammonia. In each case, only one radioactive spot was found on the paper, which corresponded exactly with the location of the β-hydroxybutyric acid spot.

Cell-free extracts of *R. rubrum* also rapidly incorporate the β-hydroxybutyryl moiety of the coenzyme *A* derivative into polymer. As in *B. megaterium*, the activity is largely associated with the particulate fraction. The kinetics of incorporation are, however, complicated by the presence of an active depolymerase which is also associated with the particles. Crude extracts were prepared by sonic oscillation of cells grown anaerobically in the light. The particulate and supernatant fractions were separated by low-speed centrifuging. The former, which contained the polymer particles, was heavily contaminated with pigmented cell debris. The native polymer, when resuspended in either the supernatant fraction or buffer, underwent rapid hydrolysis to β-hydroxybutyric acid. As much as 80 per cent of the polymer disappeared after 30 min. of incubation at 33° C.

The results of an experiment in which labelled β-hydroxybutyryl-coenzyme *A* was added to a crude cell-free preparation are shown in Fig. 1. Maximal incorporation was achieved within 2 min., after which time the total carbon-14 content of the polymer rapidly decreased. The early termination of incorporation can be plausibly ascribed to exhaustion of the substrate. Thereafter, radioactivity is lost from the polymer as a consequence of the continued depolymerization. The results of a parallel experiment, in which the resuspended particulate fraction was incubated with different concentrations of substrate, are entirely compatible with this interpretation (Table 2). The identification of the carbon-14-labelled product was carried out as described above in the experiments with *B. megaterium*.

No direct activation of β-hydroxybutyric acid with adenosine triphosphate and coenzyme *A* could be demonstrated in cell-free extracts of *R. rubrum*. A small incorporation of carbon-14 into polymer was

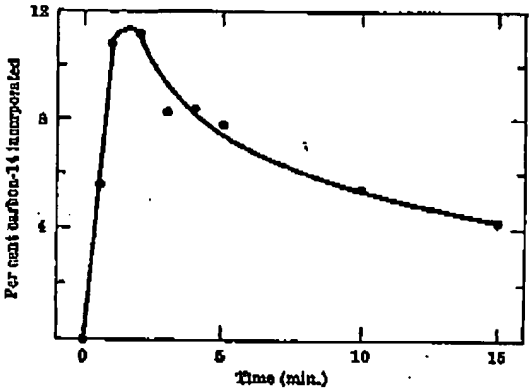

Fig. 1. Incorporation of carbon-14 into polymer by crude extracts of *R. rubrum*. The reaction mixtures contained crude extract (3·9 mgm. polymer and 5·08 mgm. protein per ml.); D(−)-β-hydroxybutyryl-coenzyme *A*, 1·4 μequiv.; and the following components (in μmoles per ml.): magnesium chloride, 12·7; glutathione, 12·7; and *tris* (hydroxymethyl) aminomethanehydrochloride buffer, *p*H 7·4, 127. The reaction mixtures were incubated at 35° C.

observed, however, when crude extracts were incubated with carbon-14-labelled acetate, adenosine triphosphate, coenzyme *A* and reduced pyridine nucleotides.

Two soluble enzymes, presumably involved in polymer metabolism, were found in the supernatant fraction of *R. rubrum* extracts prepared from cells that had depleted their polymer stores. A hydrolytic system catalyses digestion of boiled polymer particles of *R. rubrum* and native polymer particles of *B. megaterium* but does not attack the purified polymer. A specific diphosphopyridine nucleotide-linked D(−)-β-hydroxybutyric acid dehydrogenase is also present in such extracts, and has been partially purified.

Table 2. Synthesis of Polymer by Particulate Fraction of *R. rubrum* Extracts

Time (min.)	Percentage carbon-14 recovered in polymer fraction		
	Exp. 1	Exp. 2	Exp. 3
2	9	10	7
5	6	9	8
10	5	7	11

The reaction mixtures contained resuspended particulate fraction (3·1 mgm. polymer per ml.); and the following components (in μmoles per ml.): magnesium chloride, 10; glutathione, 10; and *tris* (hydroxymethyl) aminomethanehydrochloride buffer, *p*H 7·4, 100. Exps. 1, 2 and 3 contained 0·25, 1·0 and 4·0 μequiv. of D(−)-β-hydroxybutyryl-coenzyme *A* per ml. (initial), respectively. The reaction mixtures were incubated at 35° C.

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INHIBITION OF CELLULAR RESPIRATION BY CO-CARCINOGENIC FRACTIONS OF CROTON OIL

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As a result of the work of Rous and his colleagues[1] and Berenblum and his colleagues[2], it is widely accepted that carcinogenesis in skin—and probably also in other organs—takes place in two successive stages. A single small dose of a carcinogen such as 9 : 10-dimethyl-1 : 2-benzanthracene applied to mouse skin is sufficient to produce a lasting 'initiation' but no tumours. Subsequent repeated application of croton oil gives rise to a large crop of tumours, whereas croton oil applied without a previous initiating stimulus produces few, if any, tumours. Several arguments[3] make it clear that the 'promotion' or 'co-carcinogenesis' by croton oil is complementary to initiation and quite distinct from it; thus, administration of the initiator after prolonged treatment with croton oil does not lead to tumour formation.

The factor or factors in croton oil responsible for tumour promotion have not been identified, and their effects on cell metabolism have not previously been described. Lijinsky[4] has submitted methanolic extracts of croton seeds to silica-gel chromatography and obtained fractions, comprising about 1 per cent of the whole oil, that appear to have all its tumour-promoting activity. Fractions kindly provided by Dr. Lijinsky are at present being analysed by gas-chromatography in this Institute in attempts to identify the active agent or agents. In the meantime, the opportunity has been taken—for reasons which will be apparent in the discussion here—to analyse the effects of the croton oil fractions on cell metabolism. It has been found that the highly active tumour-promoting fractions are inhibitors of cellular respiration in several tissues, and this property may well be related to their co-carcinogenic effect.

Rat liver homogenates. Livers of 100-gm. hooded rats were homogenized by the Potter–Elvehjem technique in the following medium: 0·3 *M* sucrose, 0·002 *M* sodium ethylenediamine tetraacetate and 0·03 *M* nicotinamide, adjusted to *p*H 7·1 with potassium hydroxide. Each Warburg flask contained 0·3 ml. homogenate (containing 1·02 mgm. protein nitrogen per ml.), 0·2 ml. potassium succinate, 0·15 *M*, *p*H 7·1 and 1·4 ml. sucrose–ethylenediamine tetraacetate–nicotinamide as above. Croton oil fractions were added in ten-fold dilutions in 0·05 ml. ethanolic solution to give final concentrations from 0·2 to 200 μgm. per ml. Oxygen uptake from air

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APPEAL BRIEF

Evidence Appendix

1. Fukui & Doi. *J. Bacteriol.* 179:4821-4830 (1997)

2. Merrick, J.M. and Doudoroff, M. Nature 189: 890-892 (1960)

Related Proceedings Appendix

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